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STUDIES ON *MYCOBACTERIUM ULCERANS*

I. SEROLOGICAL RELATIONSHIP TO OTHER MYCOBACTERIA

by FRANK FENNER AND RONALD H. LEACH

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(Accepted for publication 7th September, 1951.)

In 1948 MacCallum, Tolhurst, Buckle and Sissons published an account of the cultural behaviour and animal pathogenicity of a mycobacterium which they had isolated from chronic ulcers of the skin in man. The microorganism was characterized by its failure to grow on the usual media used for cultivating tubercle bacilli when the cultures were incubated at 37° C. or at room temperature, but by its slow growth on these media when incubated at 33° C.; by its lack of pathogenicity for rabbits and guinea-pigs, and by the cutaneous lesions which followed its inoculation into rats and mice.

Since their original description of six cases of human infection three more cases have been recognized in Victoria (Buckle 1951), one case in Sydney (Forbes *et al.*, 1950), and one in New York, the latter from a patient infected in the Belgian Congo (van Oye and Ballion, 1950; Meleney and Johnson, 1951). In a discussion of van Oye and Ballion's paper Janssens (1950) described a series of 81 cases which he had seen amongst Africans, particularly in the children, in a relatively localized area in the Belgian Congo. There seems to be little doubt from his description that he was dealing with the same disease. Further, a series of seven cases with a similar clinical and histological picture, but from which no microorganisms were isolated, was described in Sweden (Gellerstedt, 1944). MacCallum *et al.* refrained from naming the organism in their original paper, but they have since (footnote to Fenner (1950)), proposed the name *Mycobacterium ulcerans*.

The cultural properties and pathogenic behaviour of *Mycobacterium ulcerans* seem to mark it off clearly from the other mycobacteria, although it is not inconceivable that a variant of one of the mammalian tubercle bacilli which had lost the ability to multiply at temperatures higher than 35° C. would show much the same properties. It was obviously necessary that a comparative study should be made of its antigenic structure. It was thought desirable in the first instance to use a crude serological method of comparison which might indicate group relationships, and complement-fixation with suspensions of heat-killed bacteria as the antigens was chosen as the method of study, sera

being appropriately absorbed before testing. Preliminary experiments confirmed Fürth's (1926) view of the superiority of the complement-fixation test over the agglutination test with mycobacteria. This paper describes the results of the serological study, and in the following paper the skin reactivity of guinea-pigs sensitized with various mycobacteria will be described.

MATERIALS.

Bacterial strains.

Mycobacterium tuberculosis var. *hominis*, strain H37Rv
Mycobacterium tuberculosis var. *bovis*, strain Ravenel
Mycobacterium tuberculosis var. *bovis*, strain B.C.G.
} kindly supplied by Dr. R. J. Dubos.

Mycobacterium muris, strain OV164, kindly supplied by the Commonwealth Serum Laboratories, Melbourne, Australia.

Mycobacterium avium, National Collection of Type Cultures, England, No. 7091.

Mycobacterium ranae, kindly supplied by Dr. R. J. Dubos.

Mycobacterium phlei, National Collection of Type Cultures, England, No. 525.

Mycobacterium ulcerans, strains SF, RS, RT and KR, kindly supplied by Miss J. Tolhurst, Alfred Hospital, Melbourne.

Culture media.

1. Slants of Loewenstein's medium were used for preparation of the immunizing inocula.
2. Tween-albumin liquid medium (Dubos and Middlebrook, 1947) was used for preparation of the bacillary suspensions, 100 ml. in 250 ml. Erlenmeyer flasks.
   To obtain dispersed growth of *Mycobacterium phlei* and *Mycobacterium ranae* the concentration of Tween 80 was increased from the usual 0·05 p.c. to 0·5 p.c. and albumin was omitted from the medium in which these strains were grown.
3. Incubation was carried out at 33° C. for *Mycobacterium ulcerans* strains and 37° C. for all other strains, for periods varying from 1–8 weeks, according to the strain.

Experimental animals.

Rabbits bred at the Walter and Eliza Hall Institute and weighing 1,500–2,000 gm. were used for the production of immune sera. Two rabbits were immunized with each strain of mycobacterium examined.

METHODS.

Production of antisera.

An initial sample of serum was obtained from each animal by cardiac puncture, and preserved at 4° C., after the addition of merthiolate to a final concentration of 1/10,000.

Mycobacteria which had been grown on Loewenstein slopes were carefully scraped off with a spatula, dried in an evacuated desiccator over calcium chloride, and then killed in an oven at reduced pressure at 60° C. for an hour. Suspensions of the organisms in paraffin or saline were prepared by thoroughly grinding them in an agate mortar, and slowly adding the paraffin or saline.

The rabbits were inoculated at each of four subcutaneous sites with 1 mg. of the dried, heat-killed mycobacteria suspended in 0·5 ml. of paraffin oil. A month later each animal received three intraperitoneal inoculations of a suspension of 1 mg. of the dried bacteria in saline at intervals of two days. They were bled from the heart one week after the last injection. The two sera produced against each strain were tested and the most potent one used for all subsequent tests.

Serological tests.

Antigens for complement-fixation test and for absorption.

After ensuring their freedom from contaminants, fully grown Tween-albumin cultures of the several strains of mycobacteria were deposited by centrifugation, washed once with normal saline containing 0·05 p.c. Tween 80, and resuspended in about 1/20 the volume of saline containing Tween 80 (0·05 p.c.) and merthiolate (0·01 p.c.). They were heated for an hour at 60° C. in the water bath and then allowed to stand for two days in the refrigerator. The supernatant fluid, containing well-dispersed bacteria, was removed and used, after suitable dilution, as the antigen for the complement-fixation tests. The deposit was resuspended in Tween saline and thoroughly mixed with a pipette, large clumps were thrown down by short centrifugation and discarded, and the supernatant bacilli then spun out, resuspended in a volume of saline, containing merthiolate and Tween 80, equal to three times the volume of the packed bacterial cells, and stored at 4° C. as stock absorbing suspension. Cultures of virulent mammalian tubercle bacilli, H37Rv and Ravenel, were heated at 60° C. for an hour before centrifugation, instead of after resuspension in saline, to reduce the risk of infection during handling of the cultures.

Absorption of sera.

0·4 ml. of the stock absorbing suspension (containing 0·1 ml. packed bacterial cells) was washed twice in a conical centrifuge tube, and the supernatant fluid discarded. 1 ml. of 1/10 dilution of the serum to be absorbed was then added to 0·1 ml. packed bacterial cells. This procedure was adopted to avoid the formation of soluble antibody—antigen complexes (Fürth, 1926). The serum and cells were thoroughly mixed, stood for two hours in the water bath at 37° C., with frequent agitation, and then kept overnight in the refrigerator. Next day the bacilli were spun out and the supernatant serum removed for testing by the complement-fixation test.

Complement-fixation test.

On the day of the test, all sera were inactivated at 62° C. for 15 minutes, preliminary tests having shown that this was the most satisfactory temperature for inactivation. Fresh guinea-pig serum was used as the source of complement. It was stored at 4° C. and was never used more than three days after collection. Complement was titrated daily in the presence of each antigen, and each absorbed and unabsorbed serum, as well as in saline. Both antigens and antisera were strongly anticomplementary, sometimes reducing the titre of complement by one-half. The antisera were rarely more strongly anticomplementary than the antigens and the dose of complement used in the complement-fixation tests was determined by the titre of complement in the presence of the antigen used in the test, a concentration being chosen which contained three 50 p.c. haemolytic doses.

Serum dilutions were prepared in calcium-magnesium saline (Mayer, Osler, Bier and Heidelberger, 1946) with a dropping pipette, using approximately 0·04 ml. volumes in tubes 8 cm. long and 7 mm. internal diameter. The technique followed closely that described by Donnelley (1951). Fixation proceeded for one hour at 37° C., and then one volume of 3 p.c. sheep erythrocytes, sensitized by the addition of haemolysin to a final concentration of eight 50 p.c. haemolytic doses, was added to each tube. After standing for thirty minutes at 37° C., with two periods of shaking, the test was read by visual estimation of the degree of haemolysis. The end point was taken as the tube showing 50 p.c. haemolysis. If there was no such tube the end point was estimated by interpolation. Appropriate controls were included in each titration.

Serum prepared against a given mycobacterial strain was absorbed with all the bacillary suspensions on one day, and next day the unabsorbed serum and each specimen of absorbed

serum was tested against both the homologous antigen, used in the preparation of the immune serum, and the antigen used for carrying out the absorption.

The complement-fixation antigens, prepared as outlined earlier, were standardized by opacity to a concentration of about 10^9 organisms per ml. and each was then tested by a checkerboard titration with the unabsorbed homologous immune serum to determine the optimum concentration for fixation. To conserve antigen, and to reduce the anticomplementary activity as much as possible, the optimum antigen dilution was taken as the highest dilution which gave maximum fixation with the homologous antigen. This was usually a 1/2 dilution of the stock antigen, i.e. about 5×10^8 bacteria per ml. With the *Mycobacterium ulcerans* strains RS and RT it was necessary to use the undiluted suspensions.

EXPERIMENTAL RESULTS.

As many previous workers have found, direct serological tests with unabsorbed sera, whether agglutination or complement-fixation, furnish little indication of the relationships of mycobacteria. Cross-reactions always occur. The results of direct tests on a number of sera are shown in Table 1. The highest titres were usually obtained with the homologous strain or closely related strains, but cross-reactions with quite dissimilar strains were often strong. On no occasion did fixation occur with pre-immunization sera.

The results of the tests with absorbed sera are shown in Table 2.

TABLE 1.

Results of direct complement-fixation tests with unabsorbed sera, using all antigens. The figures indicate the reciprocal of the dilution at which the 50 p.c. haemolysis occurred, using 3·50 p.c. HD. of complement. Results with homologous antigens shown in italic type.

Antiserum produced against	Antigens								
	Myco. ulcerans			Mammalian type			*Myco.*	*Myco.*	*Myco.*
	R.S.	R.T.	S.F.	H37Rv (human)	Ravenel (bovine)	OV164 (murine)	*avium*	*ranae*	*phlei*
R.S.	*60*	60	60	60	120	30	30	40	20
R.T.	120	*210*	60	120	140	53	60	60	15
S.F.	80	320	*1,280*	1,280	480	320	320	480	40
H37Rv	27	30	15	*80*	120	40	40	27	10
Ravenel	80	60	15	160	*480*	80	120	60	30
OV164	160	160	160	320	640	*640*	240	160	140
Myco. avium	240	240	240	320	960	320	*640*	140	33
Myco. ranae	60	100	60	240	240	60	60	*240*	130
Myco. phlei	480	640	640	1,280	960	240	480	960	*960*

To simplify discussion of the results, Fig. 1 was constructed on the same lines as the diagrams used by Wilson (1925) and Wells (1946). Each row of columns represents the results obtained with one immune serum, and each individual column shows the percentage reduction of antibody titre to the homologous organism, after absorption of the serum with the strain indicated at the top of the figure. The degree of absorption of each antiserum by the homologous strain is taken as 100 p.c. in each instance.

TABLE 2.

The titres of absorbed and unabsorbed antisera tested with the homologous and the absorbing antigens.

Antiserum prepared against:	Antigen for C.F. test	Absorption	Absorbing antigen ("X") RS	RT	SF	H37Rv	Ravenel	OV164	avium	ranae	phlei
RS	RS	—	60								
		+	15	10	15	30	0	30	30	40	40
	"X"	—		60	60	60	120	30	30	40	20
		+		10	5	0	0	0	0	0	0
RT	RT	—		210							
		+	20	25	40	40	17	60	53	33	60
	"X"	—	120		60	120	140	53	60	60	15
		+	0		0	0	0	0	0	0	0
SF	SF	—			1,280						
		+	30	—	15	27	—	30	10	30	60
	"X"	—	80	320		1,280	480	320	240	960	40
		+	15	—		15	—	10	10	10	15
H37Rv	H37Rv	—				80					
		+	30	40	33	0	0	0	15	15	27
	"X"	—	27	40	15		120	20	40	27	20
		+	0	0	0		27	0	0	0	0
Ravenel	Ravenel	—					480				
		+	120	120	320	15	15	160	320	320	240
	"X"	—	80	60	15	480		30	120	60	30
		+	0	0	10	15		0	15	0	0
OV164	OV164	—						120			
		+	60	40	60	15	13	5	5	15	10
	"X"	—	160	160	160	320	640		240	160	140
		+	120	60	120	30	60		40	0	15
Myco. avium	*avium*	—							640		
		+	640	320	320	240	240	20	0	160	200
	"X"	—	240	240	240	320	960	320		140	33
		+	33	15	60	27	15	15		15	13
ranae	*ranae*	—								240	
		+	80	120	120	120	80	120	60	15	60
	"X"	—	60	100	60	240	240	60	60		130
		+	0	0	0	0	15	0	0		10
phlei	*phlei*	—									960
		+	480	480	480	480	400	640	200	960	320
	"X"	—	480	640	640	1,280	960	240	480	480	
		+	120	240	160	30	480	30	20	27	

It is apparent that practically complete absorption of antibody to the two strains of *Mycobacterium ulcerans*, RS and RT, from antisera to these strains was effected by the three strains of *Mycobacterium ulcerans* used for absorption, and also by the bovine type of tubercle bacillus (Ravenel strain). No other antigen effected complete removal of the antibodies for *Mycobacterium ulcerans* from antisera prepared against this strain, although in other experiments, not recorded in this paper, BCG also absorbed all the antibody to the *Mycobacterium ulcerans* strains from sera produced against the latter. Tests with antiserum

prepared against a third strain of *Mycobacterium ulcerans*, SF, were analogous to those recorded here.

The three mammalian type tubercle bacilli showed almost complete cross-reactivity, although absorption with the vole bacillus did not remove all antibody to bovine type bacilli from antisera prepared against the latter strain. In parallel experiments with other sera, however, complete cross-reactivity was obtained between the H37Rv, BCG, and the vole bacillus. No other antigen, including the three strains of *Mycobacterium ulcerans*, was able to remove all antibody to the mammalian tubercle bacilli. The differentiation of the vole bacillus from *Mycobacterium avium*, *Mycobacterium ranae*, and *Mycobacterium phlei* by absorption of antibody from the vole bacillus antisera was by no means as clear-cut in these experiments as Wells (1946) found when he tested absorbed sera by agglutination tests.

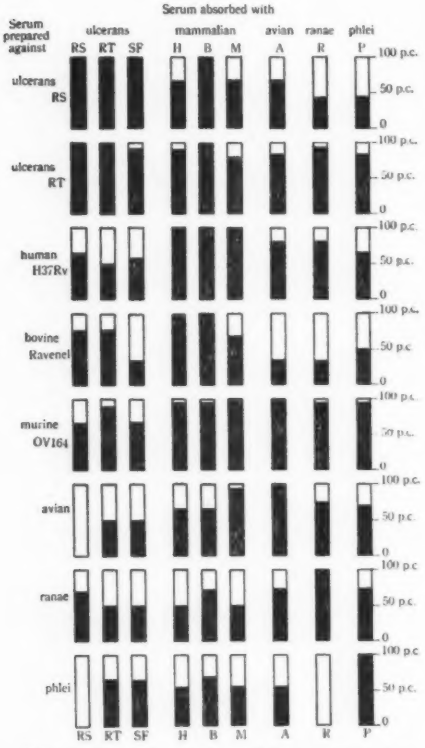

Fig. 1. The absorption of homologous complement-fixing antibody from sera to various strains of mycobacteria. Absorption by homologous strain taken as 100 p.c. and absorption by heterologous strains expressed as a percentage of the degree of absorption by the homologous strain. Data for all strains except *Myco. phlei* antiserum taken from Table 2, data for *Myco. phlei* antiserum taken from Tables 3 and 2.

The sera to each of the other three species of mycobacteria tested, *Mycobacterium avium*, *Mycobacterium ranae*, and *Mycobacterium phlei*, showed complete absorption only with the homologous antigens. There was no suggestion from these results that *Mycobacterium ulcerans* was closely related to any of these three species.

In the construction of Fig. 1 no account was taken of the relative titres of unabsorbed and absorbed sera obtained against the homologous (immunizing) strain and against that used for absorption. It may be useful to comment briefly upon these comparisons. This may be done most easily by reading down the columns ("absorbing antigen") of Tables 2 and 3.

Absorption with the RS, RT, and SF strains of *Mycobacterium ulcerans* showed that there was almost complete cross-reactivity between them. With all the other antisera, except that to the murine type bacilli, the titre to the absorbing *Mycobacterium ulcerans* antigens was not reduced to the same extent as the titre to the homologous antigens.

Absorption with H37Rv and OV164 showed much the same amount of residual antibody to *Mycobacterium ulcerans* in the sera produced against those strains as was found with the similarly absorbed *Mycobacterium avium*, *Mycobacterium ranae*, and *Mycobacterium phlei* antisera.

The bovine type bacilli, however, effected almost complete removal of antibody to both *Mycobacterium ulcerans* and bovine type bacilli from the *Mycobacterium ulcerans* antisera. When the *Mycobacterium ulcerans* antisera were absorbed with progressively smaller doses of the Ravenel strain, the reduction in titre to the homologous (*Mycobacterium ulcerans*) antigens continued to be as great as the reduction in titre to Ravenel, and complete removal of antibody to both antigens was obtained with antigen doses one-quarter as large as those necessary to effect complete absorption of the Ravenel antiserum.

TABLE 3.

The titres of antisera after absorption with large doses of antigen, tested with the homologous and absorbing antigens.

Antiserum prepared against:	Antigen for C.F. test	Absorption	Absorbing antigen ("X")					
			RS	H37Rv	OV164	*avium*	*ranae*	*phlei*
RS	RS	—	40					
		+	0	0	0	0	10	17
	"X"	—		80	60	60	60	20
		+		0	0	0	0	0
H37Rv	H37Rv	—		160				
		+	80	10	0	27	60	60
	"X"	—	27		20	60	40	40
		+	0		0	0	0	0
OV164	OV164	—			240			
		+	15	5	15	5	30	
	"X"	—	120	480		120	120	
		+	10	30		0	0	
avium	*avium*	—				320		
		+	160	33	20	0	80	
	"X"	—	120	800	480		480	
		+	20	20	0		0	
ranae	*ranae*	—					240	
		+	80	120	120	60	0	
	"X"	—	480	120	480	80		
		+	10	5	0	0		
phlei	*phlei*	—						960
		+	960	480	480	480	960	60
	"X"	—	1,280	530	480	960	480	
		+	20	0	0	0	0	

Further tests were carried out on a number of the sera, absorption being performed with twice the mass of washed bacilli used in the previous tests. After absorption for ninety minutes at 37° C., the bacilli were spun out, and the supernatant serum added to a fresh deposit of packed cells. Absorption proceeded for a further ninety minutes at 37° C., and overnight in the refrigerator. Table 3 shows the results of tests with these doubly absorbed sera, which confirmed, in the main, those recorded in the previous experiments. With both the RS and RT *Mycobacterium ulcerans* antisera, however, all antibody to the homologous as well as the absorbing antigens was removed with H37Rv, vole, and avian antigens, as well as with RS antigen. The clear-cut differences of *Mycobacterium phlei* from the other organisms are shown better when the potent *phlei* antiserum was subjected to this double absorption.

DISCUSSION.

Consideration of the three tables leads to the same conclusions. The human and bovine type strains of mammalian tubercle bacilli are closely related, as judged by cross absorption, and are clearly distinct from *Mycobacterium avium*, *Mycobacterium ranae*, and *Mycobacterium phlei*, each of which is different from all the other species tested. This confirms the results obtained by Wilson (1925) by absorption of agglutinins, and shows that the complement-fixation test as used in these experiments demonstrates the same crude relationships as does the agglutination test. Somewhat anomalous results were obtaind with antisera to the murine type bacilli. Although there were some irregularities, the strains of *Mycobacterium ulcerans* behaved in a comparable manner throughout. Antigenically they are quite distinct from *Mycobacterium avium*, *Mycobacterium phlei* and *Mycobacterium ranae*, as judged by complement-fixation tests with heat-killed bacilli, but they show a closer relationship to the mammalian tubercle bacilli. In particular, bovine type bacilli absorbed antibody to *Mycobacterium ulcerans* as efficiently as antibody to bovine type bacilli from the *Mycobacterium ulcerans* antisera, although there were clear differences in the absorption of antibodies to *Mycobacterium ulcerans* and bovine type bacilli when antisera to the latter were absorbed with *Mycobacterium ulcerans* antigens. Clarification of this relationship will have to await more detailed comparisons of the antigens of *Mycobacterium ulcerans* and *Mycobacterium tuberculosis var. bovis*. It is of some interest, however, that both Fisher's (1951) results, and cross-sensitization tests in guinea-pigs (Fenner, 1951) distinguish clearly between bovine type tubercle bacilli and *Mycobacterium ulcerans*. Repeated absorbing doses of H37Rv and OV164 (and *Mycobacterium avium*) also effected complete or almost complete removal of antibody to *Mycobacterium ulcerans* from antisera to those strains.

Mycobacterium ulcerans thus appears to be more closely related to the mammalian type tubercle bacilli than to the other mycobacteria, but is antigenically distinct from them. This observation confirms the view of MacCallum, Tolhurst and Buckle that the characters of *Mycobacterium ulcerans* are sufficiently distinctive for it to be granted the status of an independent species of the genus *Mycobacterium*.

Fisher (1951) recently reported that sera from rabbits immunized with *Mycobacterium ulcerans* (strains S.F. and K.R.) gave haemolytic titres, with red blood cells sensitized with an extract from human type tubercle bacilli, comparable to the titres of sera from animals immunized with other mycobacteria. The addition of an extract from *Mycobacterium phlei* to the system inhibited eight and sixteen units of haemolytic antibody, whereas the addition of *phlei* extract to sera produced against the mammalian and avian tubercle bacilli produced little or no inhibition of haemolysis. This isolated test suggested that, at least as far as the erythrocyte-adsorbable antigens are concerned, *Mycobacterium ulcerans* resembled *Mycobacterium phlei* more closely than the mammalian and avian tubercle bacilli. Neither the complement-fixation tests, nor tests for cross-reactivity in guinea-pigs, reported in the following paper, suggested that there was any close relationship between *Mycobacterium ulcerans* and *Mycobacterium phlei*. We hope to clarify the position by further experiments with extracts from *Mycobacterium ulcerans*.

SUMMARY.

Immune sera were prepared in rabbits against three strains of *Mycobacterium ulcerans*, against human, bovine and murine types of mammalian tubercle bacilli, and against *Mycobacterium avium*, *Mycobacterium ranae*, and *Mycobacterium phlei*.

By means of complement-fixation tests with absorbed and unabsorbed sera, using heat-killed washed bacilli as the antigens, the cross relationships between these mycobacteria were studied.

Mycobacterium ulcerans is antigenically distinct from all the other species of mycobacteria examined, although complement-fixation tests with heat-killed bacteria suggest that it is more closely related to the mammalian tubercle bacilli than to other mycobacteria.

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STUDIES ON *MYCOBACTERIUM ULCERANS*

II. CROSS-REACTIVITY IN GUINEA-PIGS SENSITIZED WITH *MYCOBACTERIUM ULCERANS* AND OTHER MYCOBACTERIA.

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Study of the serological relationships of *Mycobacterium ulcerans*, the mycobacterium recently described by MacCallum *et al.* (1948), showed that it was serologically distinct from the other pathogenic and saprophytic mycobacteria (Fenner and Leach, 1951). Although it showed cross-reactivity with all the mycobacteria it did not have the same close relationship with the other mammalian tubercle bacilli (human, bovine, and murine types) as these organisms showed one with another.

MacCallum (1948) found that some of the patients suffering from infection with *Mycobacterium ulcerans*, including a two and a half year old child with no signs of tuberculosis, gave a positive reaction to the Mantoux test with Old Tuberculin. In order to help assess the significance of this finding, and to aid in the study of the antigenic relationships of *Mycobacterium ulcerans*, investigations were carried out with several strains of mycobacteria to determine their cross-reactivity in skin tests of guinea-pigs. In this preliminary study, suspensions of washed bacilli were used as the testing antigens, as standardization of such suspensions was simpler than standardization of tuberculins for the diverse mycobacteria investigated. The results which are reported here show that, in respect of skin reactivity, the various strains of *Mycobacterium ulcerans* constitute a homogeneous group which differs from the other mycobacteria in its cross-reactivity, although as in the serological tests, they appear to be somewhat more closely related to the mammalian tubercle bacilli than to the other mycobacteria tested.

MATERIALS.

Bacterial strains.

The origin of the strains of mycobacteria used is set out in the previous paper (Fenner and Leach, 1951), except that the strain of *Mycobacterium avium* used was Camden No. 3, from the collection of Dr. R. J. Dubos, and *Mycobacterium paratuberculosis* strain TEPS was provided by Mr. J. R. Garnet, of the Commonwealth Serum Laboratories, Melbourne.

Culture media.

Sensitizing suspensions were obtained from growth on egg-slants, and testing suspensions from Tween-albumin cultures. The times and temperatures of incubation were similar to

those described in the previous paper. A heat-killed preparation of *Mycobacterium paratuberculosis*, grown as a pellicle on Dorset and Henley's medium, was used for the preparation of both sensitizing and testing suspensions.

Experimental animals.

Female albino guinea-pigs of the stock of the Rockefeller Institute, New York, each weighing 400-500 gm. at the beginning of the experiments, were used. In subsidiary experiments animals of mixed colour and sex, bred in the animal house of the Walter and Eliza Hall Institute for Medical Research, Melbourne, were used.

Methods.

Sensitization of guinea-pigs.

Mycobacteria which had been grown on the egg-slants were carefully scraped off with a spatula and heated in a vacuum oven over calcium chloride for four hours at 60° C. A suspension in sterile liquid paraffin of 1 mg. per ml. of the dried organisms was made by grinding in an agate mortar and pestle, and each guinea-pig was inoculated intraperitoneally with 0·5 ml. of the appropriate suspension.

Preparation of antigens for skin tests.

Material for skin testing was prepared by growing the required organisms in the Tween-albumin liquid medium. After exclusion of any contaminating bacteria, the dispersed organisms were deposited by centrifugation, washed with normal saline containing 0·05 p.c. Tween 80 recentrifuged, and resuspended in normal saline containing 0·05 p.c. Tween 80. The suspensions were allowed to stand overnight in the refrigerator in order to remove the coarser clumps, and the supernatant material was withdrawn and used for skin tests. The final suspensions were standardized with the "Coleman Junior" spectrophotometer, using light of wavelength 650A.U., to an optical density (on the Coleman scale) of 0·43. All bacterial suspensions were held in the refrigerator, and fresh aliquots withdrawn after thorough mixing for each series of tests, precautions being taken to avoid the introduction of contaminants.

Solutions of mammalian tuberculin purified protein derivative, P.P.D.-S. (Sharp and Dohme) and avian tuberculin P.P.D. [kindly supplied by Dr. Florence Seibert—batch P.P.D.-62 (1938)] were prepared in normal saline so that 0·0005 mg. of tuberculin was contained in the 0·1 ml. test dose.

EXPERIMENTAL RESULTS.

Cross-reactivity between four strains of Mycobacterium ulcerans.

The first experiment consisted of examination of the degree of cross-reactivity between the four strains of *Mycobacterium ulcerans*. In this experiment both the sensitizing and testing suspensions consisted of heat-killed bacilli, the former comprising bacteria grown on egg slopes and the latter consisting of saline suspensions of bacteria grown in Tween-albumin medium, washed and suspended in Tween-saline, and heated to 60° C. for 1 hour in the water bath. The interval between sensitization and testing was five weeks. On the first day, three intracutaneous inoculations of 0·1 ml. of various dilutions of selected testing suspensions were made on the left flank of the guinea-pigs, readings being made at 24 and 48 hours. Two days after the first test, three inoculations of various dilutions of another strain were made on the right flank. The results are summarized in Table 1.

TABLE 1.

Cross-reactivity of guinea-pigs sensitized and tested with various strains of Mycobacterium ulcerans. *Figures in italic type indicate reactions with homologous antigens.*

Tested with *Mycobacterium ulcerans* strain:	Sensitized with *Mycobacterium ulcerans*; strain: R.S. G.P. No. 213	R.T. G.P. No. 216	K.R. G.P. No. 218	K.R. G.P. No. 219	S.F. G.P. No. 222	S.F. G.P. No. 220	S.F. G.P. No. 221	Nil controls
R.S.	*20,7,2*++*	17,7,4,+	15,6,2,++		15,10,5,+			5,0,0,±
	10,4,0,++	10,6,2,++	10,6,0,++		13,7,4,++			2,0,0,—
R.T.	20,10,3,++	*16,7,2*,+		20,12,6,+		17,11,6,+		3,0,0,±
	10,4,0,+	*10,5,2*,++		17,7,2,++		13,6,3,+		2,0,0,—
K.R.				*20,10,4*,+			17,8,2,+	5,0,0,—
				12,4,2,++			13,6,3,++	2,0,0,—
S.F.	18,7,2,++		17,10,5,+		*16,9,5*,+	*13,8,5*,+	*17,7,3*,+	5,0,0,±
	12,6,4,++		12,7,0,++		*13,6,5*,++	*11,7,2*,++	*13,5,2*,++	3,0,0,—

Upper line, 24 hour readings; lower line, 48 hour readings.

* First figure, diameter of erythema. Second figure, diameter of blanching. Third figure, diameter of livid area; all values as mm. + etc. estimated thickness of lesion.

Only the results of the tests made with the undiluted testing suspensions are recorded and duplicate results with different animals have been omitted. When the suspensions were diluted 1/5 in saline, the responses were uniform but much weaker than those shown; erythema only, 10–12 mm. in diameter at 24 hours, occurred. In two normal animals the only response to the inoculation of the undiluted suspensions was the appearance at 24 hours of small red nodules about 5 mm. in diameter. These had almost disappeared by the end of 48 hours. It is apparent from the Table that the degree of sensitization of the different animals was comparatively uniform and that strong reactions exhibiting concentric zones of erythema, whiteness and lividity (so-called "cocarde") occurred. In any particular animal the response with different testing agents was almost identical. It was impossible to distinguish any of the four strains, which may therefore be regarded as identical in their ability to produce and elicit tuberculin type sensitization. For subsequent experiments therefore, one strain, SF, was chosen as representative of the four available strains of *Mycobacterium ulcerans*.

Cross-reactivity of the Mycobacterium ulcerans, *other mycobacteria, and avian and mammalian tuberculin P.P.D.*

The design of the main experiment on the cross-reactivity of different species of mycobacteria was influenced by observations made recently by McCarter (1949), who noticed that if multiple simultaneous test inoculations were made into sensitized guinea-pigs the resulting reactions were smaller than if a single test injection had been made, and that comparisons between different test materials could be unreliable, owing apparently to interference between the several doses of antigen. To overcome this effect, which might make comparisons of the reactivity of guinea-pigs to different testing agents difficult, the following scheme, devised by McCarter and Chase (1949), was adopted. It makes due allowance for individual differences in the attained levels of sensitization. Animals sensitized with BCG, *Mycobacterium avium*, *Mycobacterium ranae*, and *Mycobacterium ulcerans* strain SF were used in pairs, two animals of each group being allotted to each vertical column of Table 2. In addition 18 toxicity controls were used, two being allotted to each vertical column of Table 2 and two being used each day to control the toxicity of all six testing suspensions. Thus any variations in the constancy of response, owing to the diurnal variations or to the development of active sensitization by reason of the successive inoculations, were controlled. 0·1 ml. of the suspension of bacteria, or of the P.P.D. solution, was inoculated intracutaneously with a short-bevelled No. 26 needle, opposite sides of the animal being used for alternate inoculations. Readings were made at 24 and 48 hours, with a dividers and millimetre rule. The degree of induration was estimated by measuring with the dividers the thickness of the pinched-up skin in the

centre of the lesion, and subtracting the thickness of the corresponding normal skin on the other side of the animal.

TABLE 2.

Day	Column 1	2	3	4	5
0	A	B	C	D	E, F
2	B	C	D	A	A
4	C	D	A	B	homologous organism
6	A	B	C	D	—

A = *Mycobacterium ulcerans*, strain SF.
B = *Mycobacterium avium*.
C = *Mycobacterium tuberculosis*, BCG.
D = *Mycobacterium ranae*.
E = avian tuberculin P.P.D.
F = mammalian tuberculin P.P.D.

The results have been summarized in Table 3, one representative animal being selected from each group. To simplify presentation, the 48-hour readings have been omitted. In no case did they lead to an interpretation of the results different from that derived from the 24-hour readings. In all cases the other animals of each pair gave a similar type of response.

A satisfactory degree of sensitization was attained in all animals, for the response to the homologous test organism was in all cases a triple concentric reaction, the diameter of the erythema varying from 15 to 20 mm.

The toxicity controls inoculated each day showed in all cases similar reactions. With the bacillary suspensions a small nodule 5–7 mm. in diameter and usually faint pink in colour was present at 24 and 48 hours. There was usually only a tiny point of erythema or no reaction at all at the site of inoculation of the tuberculins P.P.D. In all the animals subjected to a series of successive inoculations, however, there was a change in reactivity on the sixth day, when the original inoculation was repeated, although the reactions to the 2-day and 4-day inoculations consisted, as in the other control animals, of small nodules. In the sensitized animals, the reaction to the second inoculation of bacillary suspension six days after the first was in no case diminished by the successive tests but was usually slightly greater than the reaction to the first inoculation. The discrepancy was never great enough to suggest that the intermediate (2-day and 4-day) comparisons with the first reaction were not valid.

We may consider now the cross-reactivity exhibited by animals sensitized with *Mycobacterium ranae*. These guinea-pigs were all highly sensitized to the homologous organism, were insensitive to avian and mammalian tuberculin, and showed an equal low degree of sensitization to BCG, avian tubercle bacilli, and *Mycobacterium ulcerans* suspensions.

TABLE 3.

Tuberculin-type reactions in guinea-pigs sensitized with heat-killed mycobacteria suspended in paraffin oil and tested with living bacterial suspensions, and with mammalian and avian tuberculins P.P.D. Reactions observed 24 hours after test inoculations are recorded. Figures in italic type indicate reactions with homologous antigens.

Sensitized with	Column (Table 2)	Tested with *Myco. ulcerans*	*Myco. avium*	Avian P.P.D.	B.C.G.	Mammalian P.P.D.	*Myco. ranae*
Myco. ulcerans	1	*15,10,6,2*[1] (*15,10,5,2*)[2]	14,8,0,2*		12,6,0,2		
	2		13,2,1,2 (13,5,2,2)		10,4,tr,2		12,6,0,2
	3	*16,11,7,3*			16,7,3,2 (15,8,2,2)		9,3,0,3
	4	*13,9,6,3*	13,7,2,3				13,6,0,3 (15,5,0,3)
	5	*14,9,3,3*		13,0,0,1		19,13,8,1	
Myco. avium	1	8,0,0,2 (11,5,0,2)	*18,8,4,2*		8,5,0,1		
	2		*16,13,7,4* (*17,11,9,4*)		9,4,0,2		11,4,0,2
	3	11,4,0,3			11,4,0,3 (11,4,0,2)		14,5,0,2
	4	11,2,0,2	*17,10,6,3*				16,7,0,4 (15,7,0,5)
	5	9,3,0,1	*16,11,7,3*	*16,10,5,1*		6,0,0,1	
B.C.G.	1	15,3,0,3 (20,5,0,2)	17,9,4,5		*20,13,5,3*		
	2		16,3,0,4 (12,7,4,4)		*19,12,6,3*		9,3,0,2
	3	12,7,3,3			*16,11,6,3* (*17,10,7,4*)		9,3,0,1
	4	7,0,0,1	8,3,0,1				17,0,0,1 (11,0,0,1)
	5	10,4,0,1		10,0,0,1	*15,7,3,3*	*21,13,3,1*	
Myco. ranae	1	8,5,0,2 (9,4,0,2)	10,5,0,2		7,4,0,1		
	2		11,4,0,1 (9,3,0,1)		7,4,01		*16,11,7,2*
	3	9,4,0,2			11,4,0,2 (13,3,0,2)		*20,14,9,3*
	4	6,0,0,1	9,3,0,2				*16,12,7,5* (*19,11,7,4*)
	5	7,3,0,1		3,0,0,0		3,0,0,0	*17,14,5,2*
Controls	1	nodule 5 (12,4,0,1)	nodule 8		nodule 7		
	2		nodule 6 (10,3,0,1)		nodule 6		nodule 5
	3	nodule 7			nodule 6 (12,3,0,0)		nodule 7
	4	nodule 5	nodule 8				nodule 8 (13,3,0,2)

[1] First test inoculation with a particular bacillary suspension.

[2] Second test inoculation with that suspension, six days after the first.

* Figures indicate in millimetres. First figure, diameter of erythema. Second figure, diameter of blanching. Third figure, diameter of livid area. Fourth figure, thickness of pinched-up skin minus thickness of control pinched-up skin.

Guinea-pigs sensitized with BCG organisms were highly sensitive to the homologous strain and to mammalian tuberculin P.P.D. Their cross-reactivity was greater than that of animals sensitized with *Mycobacterium ranae*, reactions to *Mycobacterium ulcerans* being greater and those to the avian tubercle bacilli being definitely larger than those seen in the animals sensitized to *Mycobacterium ranae*. The reaction to avian tuberculin P.P.D. was, however, weak.

The animals sensitized with *Mycobacterium avium* were highly sensitive to the homologous strain and to avian tuberculin P.P.D. They were moderately sensitized to all the other bacterial strains used for testing, the reactions to *Mycobacterium ranae* being perhaps slightly greater than those to the other two organisms. The reaction to mammalian tuberculin P.P.D. was weak.

Subsequent to the performance of these experiments, Fisher (1951) published the results of tests of the haemolysis by antisera to various mycobacteria of erythrocytes to which an antigen from human type tubercle bacilli had been absorbed. Rabbit antisera to two strains of *Mycobacterium ulcerans* (SF and KR) gave high haemolytic titres. When the degree of inhibition of this haemolysis by an extract from *Mycobacterium phlei* was tested, the *Mycobacterium ulcerans* sera gave results quite different from those given by the mammalian tubercle bacilli, and Fisher concluded that with regard to the erythrocyte-adsorbable antigenic material, *Mycobacterium ulcerans* occupied a place between *Mycobacterium paratuberculosis* and *Mycobacterium phlei*, and was clearly differentiated from the mammalian type bacilli. An experiment was therefore carried out to determine the cross-reactivity of guinea-pigs sensitized to *Mycobacterium ulcerans*, *Mycobacterium phlei*, and *Mycobacterium paratuberculosis*. BCG-sensitized animals were also included in the experiment. The design of the experiment differed from that just described in that both sensitizing and testing suspensions were made up with heat-killed bacilli, and the test was carried out in two stages, three antigens being inoculated on the first day and the fourth two days later. The order of inoculation of the different antigens was varied in each of the four pigs used for each group, and comparable results were obtained in all cases. Normal control animals gave negligible reactions with all inocula. The results are recorded in Table 4.

TABLE 4.

Tuberculin-type reactions in guinea-pigs sensitized with heat-killed mycobacteria suspended in paraffin oil and tested with saline suspensions of the heat-killed bacilli. Reactions observed 24 hours after test inoculations are recorded. Figures in italic type indicate reactions with homologous antigens.

Tested with	Sensitized with *Myco. ulcerans*	*Myco. paratuberculosis*	*Myco. phlei*	B.C.G.
Myco. ulcerans	*31,23,17,2*	24,14,8,4	7,0,0,1	20,7,0,5
Myco. paratuberculosis	8,0,0,1	*23,17,9,4*	10,6,0,1	13,4,0,1
Myco. phlei	11,0,0,1	13,5,0,2	*19,13,6,3*	10,0,0,1
B.C.G.	15,10,2,2	..	16,10,0,2	*26,10,4,4*

TABLE 5.

Summary of cross-reactions between different species of mycobacteria. Symbols in parenthesis indicate reactions with homologous antigens.

Tested with	Sensitized with B.C.G.	*Myco. avium*	*Myco. ranae*	*Myco. phlei*	*Myco. paratuberculosis*	*Myco. ulcerans*
B.C.G.	(+++++)	++	++	++	++	++
Mammalian P.P.D.	(+++++)	+	—	..	..	+++++
Myco. avium	+++	(+++++)	++	..	..	++
Avian P.P.D.	+	(+++++)	—	..	..	+++
Myco. ranae	++	+++	(+++++)	..	..	+++
Myco. phlei	++	..	..	(+++++)	++	++
Myco. paratuberculosis	+++	..	..	++	(+++++)	++
Myco. ulcerans	++	++	+	+	+++++	(+++++)

— to +++++ different degrees of severity of tuberculin-type reaction.

.. test not made.

All animals were satisfactorily sensitized. The results with BCG-sensitized guinea-pigs paralleled those obtained earlier, with the additional features that both *Mycobacterium paratuberculosis* and *Mycobacterium phlei* produced weak reactions. Animals sensitized with *Mycobacterium phlei* reacted strongly only to the homologous organism. Animals sensitized with *Mycobacterium paratuberculosis*, however, reacted as strongly to the suspension of *Mycobacterium ulcerans* as to the homologous strain, but did not react strongly with *Mycobacterium phlei*.

We may now consider the reactions of *Mycobacterium ulcerans* to all the antigens tested in the two experiments. In the first experiment, the guinea-pigs which had been sensitized with *Mycobacterium ulcerans* showed considerable cross-reactivity. They were highly sensitized to the homologous organism and to mammalian tuberculin P.P.D., and slightly (but more than BCG-sensitized animals) to avian tuberculin P.P.D. The reactions with BCG, *Mycobacterium avium*, and *Mycobacterium ranae* were of moderate and approximately equal intensity. Guinea-pigs sensitized to *Mycobacterium ulcerans* in the second experiment reacted strongly with the homologous antigen, with about half that intensity to BCG, and weakly to *Mycobacterium paratuberculosis* and *Mycobacterium phlei*.

If the severity of the tuberculin type reaction is graded arbitrarily from + to +++++, and the results of all the experiments combined, the picture of cross-reactivity obtained is that shown in Table 5. It is evident, as one might expect, that there is a good deal less specificity when washed bacterial cells are used for testing, compared with P.P.D. tuberculins. However, *Mycobacterium ulcerans* obviously shows greater cross-reactivity with mammalian (BCG) and avian tubercle bacilli than with *Mycobacterium ranae* and *Mycobacterium phlei*. The degree of cross-reactivity with mammalian and avian cell suspensions is about the same, but the reaction of *Mycobacterium ulcerans* sensitized animals to mammalian tuberculin P.P.D. was always much stronger than that to avian tuberculin P.P.D. On the other hand, their reactivity to avian tuberculin P.P.D. was definitely greater than the cross-reactions of either the guinea-pigs sensitized with BCG or with avian tubercle bacilli to the heterologous tuberculins.

DISCUSSION.

Taken in conjunction with the results reported in the previous paper, the distinctive nature of *Mycobacterium ulcerans* is clear. The specific status proposed by MacCallum, Tolhurst and Buckle (Fenner, 1950), appears to be fully justified by this study of the antigenic relationships of the mycobacteria. There was no indication, in either serological or skin tests, of a close antigenic relationship between *Mycobacterium ulcerans* and *Mycobacterium phlei*, such

as was suggested by Fisher's (1951) results with the erythrocyte-adsorbable antigens indicated. No explanation can be suggested for the strong reactivity to suspensions of *Mycobacterium ulcerans* of guinea-pigs sensitized with *Mycobacterium paratuberculosis*, and the lack of a reciprocal cross-reactivity.

The occurrence of strongly positive tuberculin reactions with mammalian P.P.D. in guinea-pigs sensitized with *Mycobacterium ulcerans* suggests that in man also, a positive Mantoux test may indicate infection with *Mycobacterium ulcerans* rather than *Mycobacterium tuberculosis*.

SUMMARY.

The skin reactivity of guinea-pigs sensitized with heat-killed mycobacteria of six different species was studied. Animals sensitized with *Mycobacterium ulcerans* showed strong reactivity to suspensions of the homologous organism and some cross-reactivity with other mycobacteria, especially mammalian tubercle bacilli. They also reacted strongly to the inoculation of mammalian but not avian P.P.D. tuberculin.

Animals sensitized with mammalian tubercle baccili (BCG) reacted to *Mycobacterium ulcerans*, but to a lesser degree than the reverse relationships.

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ACUTE ANURIA IN RATS

EXTRACELLULAR ELECTROLYTE STUDIES FOLLOWING BILATERAL NEPHRECTOMY[1]

by D. A. COATS[2] AND M. L. WELLBY

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Acute anuria has long been known to be a condition associated with various electrolyte changes in the extracellular fluid, yet investigations of these changes in experimental animals have not usually been comprehensive. Atchley and Benedict (1927) studied the changes occurring in dogs following ureteral ligation, of phosphate, sulphate, chloride, carbonate and total base, and found profound changes in their distribution, with an almost equimolecular interchange of a rise of phosphate and sulphate, and a fall of chloride and carbonate. Hoff, Smith and Winkler (1941) studied changes in extracellular potassium levels in dogs rendered anuric by ureteral ligation, bilateral nephrectomy and intravenous injection of mercuric chloride, and correlated these changes with survival time and electrocardiographic changes, concluding that in their animals hyperkalemia was the essential cause of death. Much more recently Harris, McDonald and Williams have studied in detail the electrolyte changes occurring in the extracellular fluid in sheep following ligation of both ureters.

The present study of the extracellular electrolyte pattern in rats has been undertaken in an attempt to determine:

(i) The nature and extent of alterations in the extracellular electrolyte pattern following bilateral nephrectomy.

(ii) The extent to which these alterations can be modified by depression of protein metabolism with a protein-free high-calorie diet.

(iii) Whether the survival time of the anuric animals is prolonged by depression of protein metabolism without significant modification of electrolyte changes.

(iv) If any modifications in the electrolyte changes are produced by depression of protein metabolism, whether these modifications can be correlated with the survival time of anuric animals.

The rate of rise in blood urea levels was used throughout in the nephrectomized animals as an index of protein metabolism.

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[1] This work was supported by a grant from the National Health and Medical Research Council.

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Rats were chosen for this experiment for several reasons: (i) like the human they are omnivorous and in a general way their normal metabolism is similar, except that it proceeds at a greater rate (Donaldson, 1924); (ii) they have a standard normal extracellular electrolyte pattern with little individual variation (Donaldson, 1924); (iii) their small size makes them easier to handle in suitable numbers than larger animals. As experimental animals for this study they had one definite disadvantage, in that the volume of blood required for the several estimations, relative to their total blood volume, was so large that it was not possible to carry out serial estimations on any one animal and in some cases it was impossible to repeat estimations where for some reason the original estimation was misplaced. This disadvantage was to some extent compensated by the fact that with small animals it was possible to use a larger series than would have been the case with larger animals. The animals used were all hooded rats, bred from the one strain and kept under the same conditions on the same mixed diet, prior to use. Both males and females were used, their weights ranging from 150 to 300 gm.

The rats were divided into six groups, two of which were subjected to bilateral nephrectomy and four of which were virtually controls:

(i) Group A. Bilateral nephrectomy was performed, and the animals were fed subsequently *ad libitum* on the same normal mixed diet as the whole colony.

(ii) Group B. Bilateral nephrectomy was performed, and the animals were fed subsequently on a diet of 100 calories per day, protein free, consisting of 16 gm. of glucose and 4 gm. of peanut oil, bound and made into cubes with paraffin wax. The rats were kept in separate cages and observation showed that they consumed this ration completely for about 48 hours after operation. The animals living longer than this took very little food.

(iii) Group C. A sham operation consisting of a left nephrectomy and the removal of a piece of muscle from the right thigh was performed, and the animals were fed subsequently a normal diet as in Group A.

(iv) Group D. The same sham operation was performed as in Group C, and the animals were fed subsequently the same special diet as in Group B.

(v) Group E. Unoperated animals, fed a normal diet, as in Group A.

(vi) Group F. Unoperated animals, fed the same special diet as in Group B.

There was no restriction of fluid intake in any of the six groups.

Each group of animals was then subdivided into five subgroups, and the extracellular ionic pattern was investigated 14, 28, 42, 56 and 70 hours from the commencement of the experiment. In this way estimations were carried out on a total of 112 animals.

Technical Methods.

All operations were carried out under ether anaesthesia. In performing the nephrectomy the adrenal was carefully preserved and, where necessary, dissected away from the kidney before the latter was removed. A single silk ligature was used to tie the renal pedicle.

Blood for estimations was taken by cardiac puncture through the chest wall, the animals being anaesthetized. Usually the volume of blood taken (5–10 ml.) was so large that the animal died, but when an animal did survive it was not used again.

pH of whole blood, after temperature equilibration, was estimated with a Jones Model B

pH electrometer and corrected to 38° C. by use of the formula $pH_{38} = pH_t - 0{\cdot}0147\ (38 - T)$ where T is the temperature in degrees centigrade of the blood sample at the time of reading.

Plasma protein estimations, were made using the copper sulphate method (Phillips, Van Slyke, Dole, Hamilton and Archibald, 1945).

Urea was estimated on 0·2 ml. whole blood using the urease-Nesslerisation method of Archer and Robb (Harrison, 1944).

Sodium was estimated in plasma in the first half of the series by the gravimetric pyroantimonate method (Kramer and Tisdall, 1921) and in the second half of the series using the Beckman oxyacetylene flame spectrophotometer. Agreement between the two methods was within 1–2 p.c.

Potassium was estimated in plasma in the first half of the series by a colorimetric modification of the cobalti-nitrite method (Jacobs and Hoffman, 1931) and in the second half of the series using the Beckman oxyacetylene flame spectrophotometer. Duplicate samples estimated by the two methods gave readings for the colorimetric method 0·5–1·0 m.eq./litre higher than with the flame spectrophotometer.

Chloride estimations were performed on 0·2 ml. plasma by the method of Schales and Schales (1941).

Bicarbonate estimations were performed on 0·2 ml. of plasma by a micro modification of the titration method of Van Slyke (1922), the back titration with sodium hydroxide to the original pH of the plasma being determined by the use of the Jones pH electrometer.

Sulphate estimations were performed on 0·5 ml. of a plasma filtrate prepared by diluting the plasma 1 in 5 with trichloracetic acid. A modification of the method of Cuthbertson and Tompsett (1931) was used, alcohol being used throughout instead of acetone, as suggested by Power and Wakefield (1938).

Phosphate estimations were performed on 3·0 ml. of the same trichloracetic acid filtrate, by the method of Fiske and Subbarow (1925).

RESULTS.

Control animals.

There was no statistically significant difference between the four control groups for the plasma concentrations of bicarbonate, chloride, sulphate, phosphate, sodium or potassium, and therefore the results on these four groups have been considered together as constituting normal control values for the animals used. There were statistically significant differences observed in the blood urea and blood pH values in the four control groups (Figs. 1, 9). The blood urea level for unoperated animals on a non protein high-calorie diet ($39{\cdot}44 \pm 4{\cdot}43$ mg./100 ml.) was significantly lower than the blood urea levels for each of the other three control groups ($64{\cdot}56 \pm 4{\cdot}43$, $56{\cdot}00 \pm 4{\cdot}43$ and $57{\cdot}11 \pm 4{\cdot}43$ mg. urea/100 ml. in groups C, D, and E respectively—in each case $P < 0{\cdot}001$). The blood pH for sham operated animals on a non protein diet ($7{\cdot}47 \pm 0{\cdot}03$) was higher than for sham operated animals fed *ad libitum* ($7{\cdot}37 \pm 0{\cdot}03$). These significant differences between the respective means were demonstrated by the "t-test" with a pooled estimate of variance with 70 and 71 degrees of freedom respectively, the significance being less than 0·001 and 0·02 respectively.

There was no significant difference between the values for plasma protein in any of the four control groups.

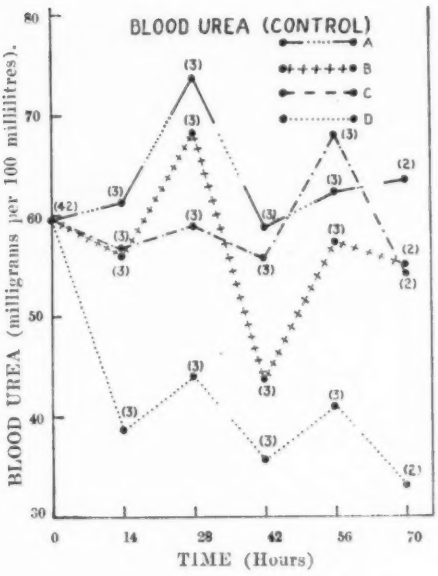

Fig. 1

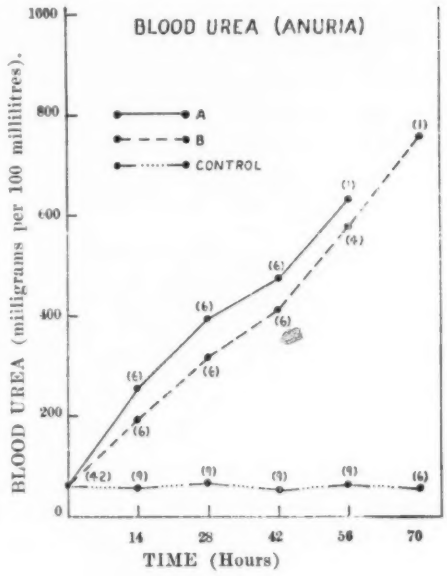

Fig. 2

Fig. 1. Mean blood urea levels for control groups (significance of groups A, B, C and D as in text). The numbers in brackets refer to the numbers of animals used for each mean value plotted.

Fig. 2. Mean blood urea levels for nephrectomized animals. The numbers in brackets refer to the number of animals used for each mean value plotted. A and B as in text.

Bilaterally nephrectomized animals.

Blood urea. The blood urea levels of the nephrectomized animals rose rapidly and in a more or less rectilinear fashion from a normal level of 59·69± 1·21 mg./100 ml. There was a significant difference between the rate of blood urea rise in the two dietary groups of animals (Fig. 2), those fed *ad libitum* showing blood urea levels of 471·33±31·81 mg./100 ml. in 42 hours compared with 410·83±31·81 mg./100 ml. in animals fed a non protein diet, (coefficients of regression 10·689 and 9·161 mg. urea/100 ml. per hour respectively, with P less than 0·05). The highest blood urea level recorded was 759 mg./100 ml., occurring in the only animal of the series which survived 70 hours after bilateral nephrectomy. This depression of the rate of blood urea rise where protein metabolism is decreased by a protein-free, high-calorie diet is in accord with findings of other workers (Borst, 1948; Bull, Joekes and Lowe, 1949).

Electrolyte balance. Alterations of the individual serum electrolyte values were of a fairly large order, and chemical equivalence was demonstrated between

total anion and total cation changes. There was some change in the total serum electrolyte concentration, the net effect at 56 hours being a total serum electrolyte deficit of 16 milliosmols per litre, as compared with the normal control animals (Table 1).

TABLE 1.

Ion	Anion rises or cation falls[1] (milliequivalents/litre)	Anion falls or cation rises[1] (milliequivalents/litre)
$HCO3'_3$	—	14
Cl'	—	18
SO_4''	11	—
HPO_4''	12	—
$K+$	—	4
$Na+$	12	—
Total	35	36

[1] mean 56 hour figures.

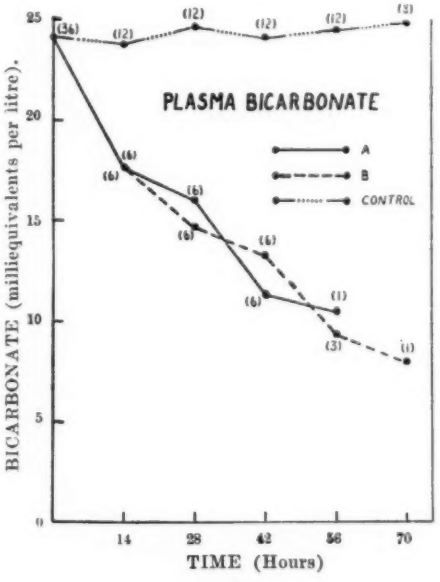

Fig. 3

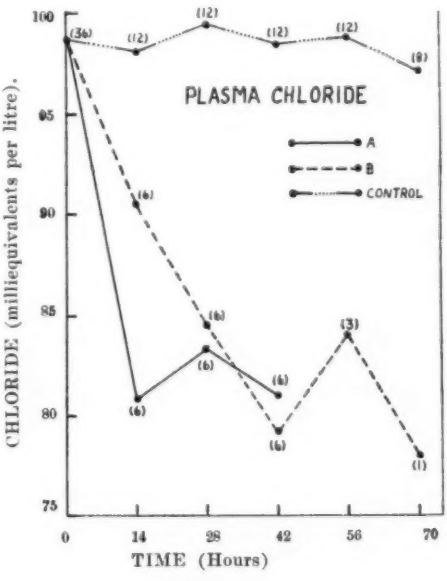

Fig. 4

Fig. 3. Mean plasma bicarbonate levels. The numbers in brackets refer to the number of animals used for each mean value plotted. A and B as in text.

Fig. 4. Mean plasma chloride levels. The numbers in brackets refer to the number of animals used for each mean value plotted. A and B as in text.

Bicarbonate. Plasma bicarbonate levels fell progressively following bilateral nephrectomy, there being no difference between the groups of animals on the different diets (Fig. 3). There was no significant alteration in the plasma bicarbonate levels of the sham operated animals, whereas Harris, McDonald and Williams recorded a rise in plasma bicarbonate and a fall in plasma chloride in sheep within a few hours of operation. This may be a species difference in the two experimental animals, or may be due to the fact that in the rat the first post-operative bicarbonate estimation at fourteen hours, due to its more rapid metabolism, was beyond the time when any initial nonspecific bicarbonate rise might have been expected to occur. The fall in plasma bicarbonate was exponential, from a normal value of 24·10±0·51 m.eq./l. down to 9·63±1·25 m.eq./l. after the animals had been anuric for 56 hours.

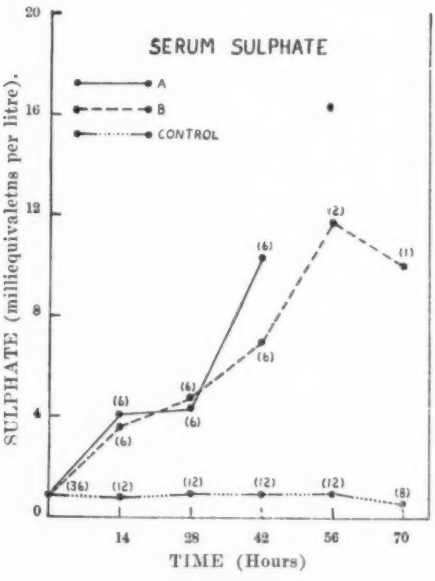

Fig. 5

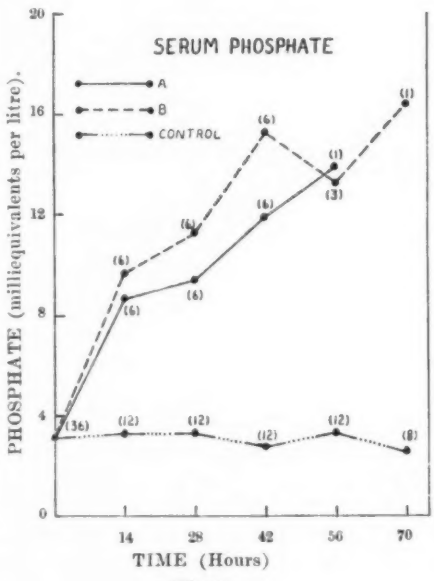

Fig. 6

Fig. 5. Mean serum sulphate levels. The numbers in brackets refer to the number of animals used for each mean value plotted. A and B as in text.

Fig. 6. Mean serum phosphate levels. The numbers in brackets refer to the number of animals used for each mean value plotted. A and B as in text.

Chloride. In all anuric animals there was a fall in plasma chloride from an initial level of 98·64±1·03 m.eq./l. down to 83·92±1·78 m.eq./l. in 28 hours, after which time the chloride fall was very much less (Fig. 4). Sham operated animals did not show any depression in plasma chloride after 14 hours, so that the sharp fall in chloride in the anuric animals in the first 28 hours is evidently specific, a result of the anuric state, and not simply due to operative interference.

Sulphate. Sham operated animals and unoperated controls did not show any alteration in serum sulphate levels. Anuric animals, however, showed a sharp rise in serum sulphate from an initial level of 0·86±0·39 m.eq./l. to 8·65±0·67 m.eq./l. in 42 hours and two animals estimated 56 hours post-operatively had serum sulphate levels of 13·7 and 9·7 m.eq./l. respectively (Fig. 5).

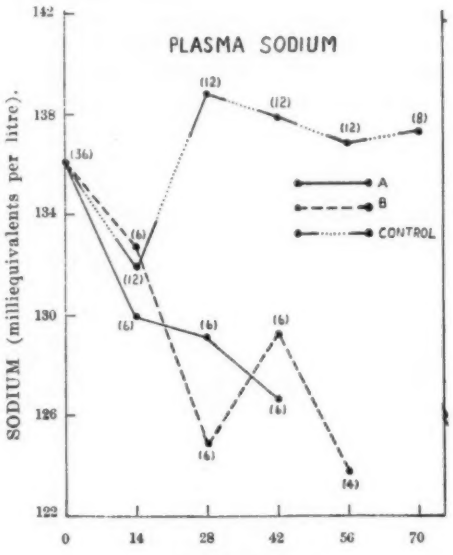

Fig. 7

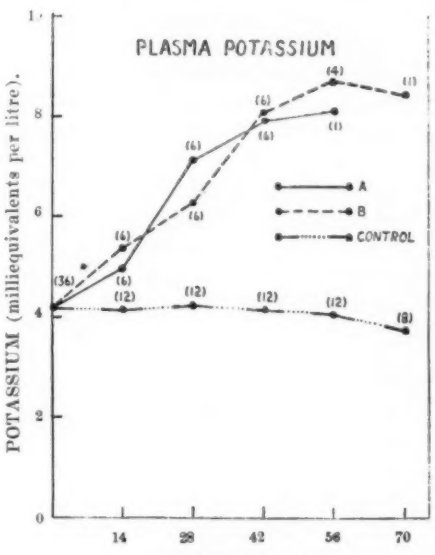

Fig. 8

Fig. 7. Mean plasma sodium levels. The numbers in brackets refer to the number of animals used for each mean value plotted. A and B as in text.

Fig. 8. Mean plasma potassium levels. The numbers in brackets refer to the number of animals used for each mean value plotted. A and B as in text.

Phosphate. There was no alteration in serum phosphate levels in sham operated animals or unoperated controls. In the anuric animals serum phosphate rose from an initial level of 3·10±0·34 m.eq./l. to 13·54±0·60 m.eq./l. in 42 hours, after which the rise was less steep. The steepest rise in serum phosphate was in the first 14 hours period of anuria (mean level 9·16±0·60 m.eq./l.) the rise throughout being curvilinear (Fig. 6). Although the accumulations of phosphate and sulphate after 56 hours anuria were of the same order, the main phosphate rise occurred earlier than the main sulphate rise.

Sodium. Although there was greater variability than in other electrolyte levels, there was no significant difference between the plasma sodium levels for the sham operated and unoperated control animals, with most figures falling in

the range 132 to 140 m.eq./l. (135·15±1·93 m.eq./l.). In both dietary groups of anuric animals there was a significant fall in plasma sodium (P less than 0·05), there being a mean fall after 56 hours of 12·37±5·80 m.eq./l. (Fig. 7). Plasma sodium and chloride concentrations fell approximately the same number of milliequivalents, but relative to the initial ratio of chloride to sodium the chloride fall was greater than that of sodium (initially Cl : Na=0·72, after 56 hours Cl : Na=0·68). Had the falls in chloride and sodium levels been due simply to haemodilution resulting from an increase in extracellular fluid volume, there would have been no alteration in the chloride to sodium ratio, and also there would have been a concurrent fall in plasma protein concentrations. This did not occur. This differential depression of chloride and sodium levels suggests that chloride, under these experimental conditions, has moved from the extracellular fluid in excess of sodium. The nature and extent of this transfer cannot be determined from the data presented.

Potassium. Sham operated animals and unoperated controls of both dietary groups showed no differences in their plasma potassium levels. Both groups of anuric animals showed a more or less rectilinear rise in plasma potassium from an initial value of 4·18± 0·11 m.eq./l. to 8·00±0·11 m.eq./l. after 42 hours, and a much more gradual rise after this time in the animals surviving longer (Fig. 8). The significance of this rise in plasma potassium as a factor in causing death of the anuric animals cannot be determined from the present experimental conditions. The rise in plasma potassium is much less than that reported by Hoff, Smith and Winkler (1941) in anuric dogs, their nephrectomized animals dying with electrocardiographic evidence of potassium intoxication and serum potassium levels of 15·0±2·00 m.eq./l. However, it is greater than that found by Harris, McDonald and Williams in sheep.

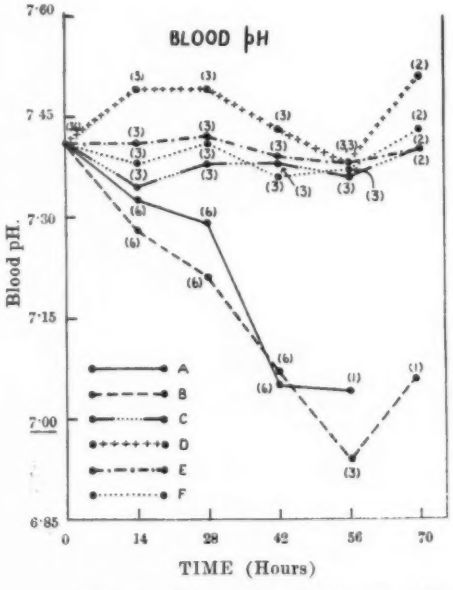

Fig. 9. Mean blood pH levels. The numbers in brackets refer to the number of animals used for each mean value plotted. A, B, C, D, E and F as in text.

Blood pH. It is possible that the whole blood pH for sham operated animals fed a high-calorie, protein-free diet was higher than in blood pH for sham operated animals fed *ad libitum* (Fig. 9), but apart from this there were no significant differences between the control groups of animals, mean pH being 7·41± 0·014. Both groups of anuric animals showed a constant and more or less rectilinear depression of blood pH down to 7·06±0·014 after 42 hours and 7·00 or lower after 56 hours (Fig. 9). Depression of blood pH was roughly proportional to the rise in serum phosphate and sulphate levels.

Plasma protein. There was no significant difference in total plasma protein in the various control groups, the mean for all control animals being 6·14± 0·069 gm. protein/100 ml. Bilaterally nephrectomized animals of both groups showed some degree of haemoconcentration as judged by plasma protein levels, but only in the group fed *ad libitum* was the rise in plasma protein statistically significant, the mean value after 42 hours' anuria being 6·52±0·169 gm. protein/100 ml. (P less than 0·05). Anuric animals fed a non-protein diet showed some rise in plasma protein, but the rise fell short of statistical significance (P greater than 0·05).

DISCUSSION.

Changes in the extracellular electrolytes in experimental anuria have not been shown, in the present work, to be modified in any significant degree by an attempt to depress protein metabolism. Feeding a protein-free, high-fat, high-carbohydrate diet resulted in a significant depression in the rate of accumulation of urea, and in so far as urea accumulation is an index of protein katabolism in anuria the dietary regime employed resulted in its depression, findings which are in accord with those of other workers (Borst, 1948; Bull, Joekes and Lowe, 1949; Kolff, 1947). In the present experiment insufficient animals were allowed to run their full course until death following nephrectomy to draw statistically valid conclusions, but it is at least suggestive that whereas four animals out of six survived 56 hours after bilateral nephrectomy and one animal out of four survived 70 hours when on the protein free dietary regime, only one animal out of six survived 56 hours and none of four survived 70 hours when fed *ad libitum,* all other factors being the same. If it be true that depression of protein katabolism increases survival time in experimental anuria, then this effect is not due to any modification of the extracellular electrolyte changes, and must be presumed to be due to some other factor or factors not yet investigated.

When the various extracellular electrolyte changes in experimental anuria are considered, disregarding the fact of the two different experimental diets, several quite constant and interesting changes are seen. There is a depression of plasma levels of the two main electrolyte components, chloride and sodium, in amounts that are roughly equimolecular but which differ greatly from the normal extracellular chloride to sodium ratio. This differential depression of chloride and sodium levels with respect to their normal ratio, together with the increase in plasma protein levels, is evidence that the movement of these

ions from the circulating extracellular fluid is real and is not merely an apparent effect due to haemodilution. Although it is clear that these electrolytes are moving from circulating plasma there is as yet no evidence as to their final location after redistribution.

Depression of blood pH is a quite constant finding in these animals, and is a reflection of the total electrolyte changes occurring in a state of metabolic anion addition. Rises in sulphate and phosphate levels were associated with falls in bicarbonate and chloride, but the observed depression of blood pH down to 7·00 or less indicates that the respiratory response to acidaemia was inadequate to compensate for increase of non-volatile acids.

A rise in plasma potassium levels following nephrectomy was also a constant finding. The significance of this change as a factor in causing death cannot as yet be assessed, though in further work still in progress anuric rats have been found to die with plasma potassium levels kept as low as 5·7 milliequivalents per litre, indicating that if potassium intoxication is a factor in causing death in anuria it is certainly not the only factor.

The various investigations undertaken have not shown any one factor to be the immediate cause of death in anuria. Hyperkalemia and pH fall have both been constantly observed, and one or both could be implicated. There is, however, some evidence suggesting that survival time may be prolonged by depressing protein metabolism. As this measure does not affect the changes in plasma potassium or blood pH, the precise significance of these factors as causes of death remains obscure.

SUMMARY.

Electrolyte changes in the extracellular fluid of rats were studied following bilateral nephrectomy.

Urea accumulation was significantly less in nephrectomized animals fed a protein-free high-calorie diet than in animals fed *ad libitum*.

There is some evidence, though insufficient for statistical significance, to suggest that survival following bilateral nephrectomy was prolonged by feeding a protein-free diet.

Animals fed *ad libitum*, and animals fed a protein-free diet, following bilateral nephrectomy, showed the same changes in the extracellular electrolytes investigated. These were rises in sulphate, phosphate and potassium levels of 11, 12 and 4 milliequivalents per litre, and falls in bicarbonate, chloride and sodium levels of 14, 18 and 12 milliequivalents per litre respectively (average values after 56 hours). Blood pH fell to 7·00 or less.

Plasma protein levels increased in anuria, suggesting some degree of haemoconcentration. In spite of this, chloride and sodium levels in plasma fell in a ratio different from their normal plasma ratio, indicating a transfer from circulating extracellular fluid.

Possible causes of death in anuria are discussed but no factor investigated emerges as being the essential cause.

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THE ELECTROLYTE PATTERN IN EXPERIMENTAL ANURIA

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Despite the fact that a gross distortion of the plasma electrolyte pattern is known to occur in anuria, there are, to our knowledge, only two comprehensive experimental studies on this aspect of the condition; those of Atchley and Benedict (1927), and those of Hoff, Smith and Winkler (1941). Acquaintance with the results of these experiments is, however, by no means common knowledge, and the changes which take place in the extracellular fluid as a result of acute renal insufficiency are still frequently misunderstood. This investigation was undertaken with a view to delineating as clearly as possible the nature and the significance of these changes in the simple experimental situation. Clinical material is frequently complicated by factors which are essentially incidental to the anuria itself, but which may none the less profoundly modify the pathological physiology of the condition. For this reason the experimental findings are reported separately; and they are, in their own right, a study of what occurs in the extracellular fluid when renal excretion is abolished. The interpretation of clinical data and the correlation of these data with the experimental are the subject of another communication.

EXPERIMENTAL PROCEDURE.

The animal used in these investigations was the sheep. This was chosen for three reasons. In the first place, the ureters can be tied in the sheep with very little trauma, using only a local anaesthetic; in this way any complications which might be introduced by the use of a general anaesthetic are avoided. Secondly, the sheep can be bled without difficulty, and enough blood collected each day for a comprehensive electrolyte analysis. Despite the repeated bleeding, the total volume of blood withdrawn over the whole period of anuria does not constitute a significant blood loss for an animal of this size. Finally, the survival time of the sheep after ligation of both ureters is long enough for full analyses to be carried out repeatedly, including muscle analyses, and estimations of both circulating and extracellular fluid volume. It may be argued that the physiological organization of the sheep is different from that of the human, and this is, of course, true; but as far as anuria is concerned, the changes which occur in the extracellular fluid of the sheep are essentially similar to those which occur in the extracellular fluid of man. For the most part, the points of difference between the two species in this respect are quantitative only, and will be discussed in some detail elsewhere.

The procedure adopted was as follows. Each animal was bled from the external jugular vein prior to operation, so that the normal electrolyte pattern for that animal could be

[1] This work was carried out with the aid of a grant from the National Health and Medical Research Council.

determined. The pre-operative circulating volume was estimated by injection of congo-red. Both ureters were then tied under local anaesthesia, and samples of muscle for water and electrolyte analyses were removed from one flank in the course of the operation. The extracellular fluid volume was determined as the inulin space; the technique used for estimating the inulin space, when both ureters are tied, is described below. After operation, samples of blood were taken each day. At least once during the period of anuria the circulating and extracellular fluid volumes were again determined, and samples of muscle taken from the opposite flank.

In some cases only one ureter was tied at the first operation, and the second tied some days later. In other cases a sham operation was carried out two or three days before the ureters were tied. Samples of the urine in the renal pelvis were obtained in one animal by tying a polythene tube into the ureter at operation, and leading it to the exterior. This tube was clamped off, and the clamp released whenever a specimen of urine was required. In all animals in which the morbid anatomy was studied the post-mortem examination was carried out immediately after death, and the tissue specimens were in fixative within a few minutes.

Chemical methods.

Analysis of the plasma. The plasma sodium and potassium were estimated by flame spectrophotometry (Wynn *et al.*, 1950). The plasma bicarbonate was calculated from total plasma carbon dioxide, as determined by the manometric method of Van Slyke and Neill (1924). Calcium was estimated by the Clark-Collip modification of the Kramer-Tisdall method (Clark and Collip, 1925); magnesium by the method of Orange and Rhein (1951); chloride by the potentiometric method of Northrop (1948); sulphate by the method of Power and Wakefield (1938); phosphate by the method of Fiske and Subbarow (1925); urea by the method of Van Slyke and Cullen (1914). The plasma protein concentration was derived from the specific gravity of the plasma; this was measured by the copper sulphate method of Van Slyke *et al.* (1945).

Analysis of the red blood cells. Sodium and potassium were estimated by flame photometry. Chloride was estimated by the Van Slyke modification of Whitehorn's method (Van Slyke, 1923).

Estimation of the circulating fluid volume and the extracellular fluid volume. The circulating fluid volume was determined by the congo-red method of Keith, Rowntree and Geraghty (1915), the concentration of dye present in the specimens being estimated spectrophotometrically. The extracellular fluid volume was determined by a modification of the inulin space method. This modification was necessitated by the fact that in these experiments both ureters were tied. The animal was bled prior to the injection of inulin, and the plasma from this bleeding was used as a blank. A known amount of inulin was then given intravenously, and two hours later the animal was again bled. The inulin space was calculated from the concentration of inulin in the plasma of this second bleeding. When the determination was to be repeated in the same animal, the amount of inulin still present in the extracellular fluid as a result of the first determination was estimated from the pre-bleeding. A known amount of inulin was again injected and the animal bled two hours later. The inulin space was calculated from the difference between the inulin concentration in the plasma of the pre-bleeding, and that in the plasma two hours after injection. The actual values given by these methods for the circulating and the extracellular fluid volumes are, of course, only approximate. However, if precisely the same technique is used for each estimation, consistent results are obtained, and the alterations which take place in these volumes during the course of an experiment lie well outside the range of experimental error.

Estimation of the haematocrit, the blood sedimentation rate and the whole blood pH. The haematocrit and the blood sedimentation rate were estimated by means of the Wintrobe tube; the blood pH was determined directly with a standard glass electrode pH meter.

Analysis of the urine. The sodium and potassium concentrations of the urine were determined by flame photometry; the chloride by the method of Volhard and Harvey (Hawk and Bergeim, 1937); ammonia by the method of Van Slyke and Cullen, as modified by Hawk *et al.* (1947); the pH with a glass electrode pH meter.

Analysis of muscle. The whole muscle water concentration was determined gravimetrically. Sodium and potassium were estimated by flame photometry after the tissue had been digested by concentrated nitric acid. Chloride was estimated by the method of Van Slyke (1923).

RESULTS.

The behaviour of the animals in this form of experimental anuria was marked by three salient features: they refused to take any food whatever; they drank only minimal amounts of water, invariably less than half a litre in twenty-four hours; and they developed some degree of diarrhoea after the fourth or fifth day. Fits did not occur, and muscular twitching, although present in some cases, was never very prominent. The survival time after ligation of both ureters varied considerably, the longest case in this series surviving ten days.

Weight. There was in all cases a progressive loss of weight during the course of the anuria. This loss varied from two to seven kg., and was greatest in those cases in which diarrhoea was marked.

Blood pH. All estimations of the blood pH were carried out on samples taken from the external jugular vein. Within a few hours of operation, there was a rise in the blood pH of 0·05 to 0·1 of a pH unit. From this raised post-operative level, the pH fell very gradually during the period of anuria, but in the uncomplicated case only to more or less the pre-operative level. In only one case did the pH fall below 7·3, and in this instance the anuria was complicated by severe diarrhoea for eight days.

Inulin space. The inulin space was grossly diminished during the course of the anuria. This diminution was most marked in diarrhoeal cases, but a fall of 20 to 30 p.c. occurred even in cases uncomplicated by diarrhoea. In one animal, which had prolonged diarrhoea, the inulin space was finally reduced to about a third of its original dimensions.

Plasma volume. The changes in inulin space were accompanied by similar changes in the plasma volume. A fall occurred in all cases, but was most marked in those with diarrhoea.

Haematocrit. In assessing the significance of the changes in haematocrit, the fall in plasma volume must be taken into account. If no other changes occurred, the amount of blood withdrawn for analyses would produce a fall in haematocrit of only 2 p.c. in ten days, so that this source of error may for practical purposes be neglected. Despite the gross diminution in plasma volume, there was, in general, a gradual fall in the haematocrit throughout the anuric period. The actual reduction in the total number of circulating red cells must therefore have been considerably greater than would be indicated by the haematocrit alone. In two animals with diarrhoea, a rise in haematocrit occurred terminally, but in these cases the diminution in plasma volume was extreme.

Blood sedimentation rate. The behaviour of the red blood cells in experimental anuria in sheep is quite different from what is found clinically in humans. Human anuria is invariably associated with a marked increase in the blood sedimentation rate. This was not the case in sheep.

Plasma analyses.

Sodium. The plasma sodium was not subject to large fluctuations. A small rise of two to four milliequivalents per litre occurred during the first or second day after the operation. In some cases a further rise of one to two milliequivalents per litre occurred during the third or fourth day. When diarrhoea supervened the plasma sodium concentration in general fell, but when the diarrhoea was associated with very marked diminution of the extracellular fluid volume, the plasma sodium concentration actually rose. Analysis of the diarrhoea fluid in these cases showed that it contained water in excess of electrolytes relative to plasma proportions.

Potassium. There was, as a rule, a gradual rise in the plasma potassium level during the period of anuria. In only one case, however, did the plasma potassium rise above 6·5 milliequivalents per litre. In this case it rose to 8·0 milliequivalents per litre during the twenty-four hours before death, but this terminal period was complicated by melaena, and the animal was found post-mortem to have gross adrenal haemorrhages. Furthermore, a significant rise in the plasma potassium concentration was not an invariable finding. In one animal, which did not have diarrhoea, the plasma potassium level on the day of death was only 4 milliequivalents per litre, the pre-operative level being 3·7 milliequivalents per litre. In another, which had some diarrhoea terminally, the potassium level on the day of death was actually lower than the pre-operative level. Whether a gradual rise to five or six milliequivalents per litre represents potassium intoxication in the sheep is uncertain. Electrocardiograms taken shortly before death in two animals with raised plasma potassium levels did not show the changes usually considered characteristic of severe potassium intoxication. These findings differ from those obtained by Hoff, Smith and Winkler (1941) in the dog. According to these investigators experimental anuria in that animal is associated with a very marked rise in the plasma potassium concentration, and with gross electrocardiographic changes.

Calcium. The changes which occurred in the plasma calcium concentration were very similar in all cases. Within a few hours after operation the plasma calcium fell 0·5 to 1·0 milliequivalents per litre. During the period of anuria a further fall occurred, but only very gradually over several days. In no case did the calcium fall below three milliequivalents per litre. The level at the time of death was usually about 1·5 milliequivalents per litre below the pre-operative level.

Magnesium. With minor fluctuations due to operative interference, there was a steady rise in the plasma magnesium concentration throughout the period

of anuria. The extent of this rise was 1·0 to 1·5 milliequivalents per litre, representing an increase of 50 to 100 p.c. over the pre-operative level.

Bicarbonate. Within a few hours of operation there was in all cases a rise in the plasma bicarbonate of three to ten milliequivalents per litre. This rise coincided with the post-operative rise in blood pH. It was produced not only by tying both ureters, but also by tying one ureter, or by sham operation alone. After the two latter procedures the plasma bicarbonate returned to its pre-operative level within three or four days. In the anuric animal, however, the raised bicarbonate was maintained until significant accumulation of sulphate and phosphate occurred. As the concentration of sulphate and phosphate in the plasma increased, so the bicarbonate gradually fell. In general it did not fall below the pre-operative level, but in one case, which was complicated by prolonged diarrhoea, the plasma bicarbonate on the day of death was 11·9 milliequivalents per litre. The sulphate concentration in this case reached 27·3 milliequivalents per litre.

Chloride. Coinciding with the rise in bicarbonate and pH, there was, in all cases, a post-operative fall in the plasma chloride concentration of three to ten milliequivalents per litre. In some cases the extent of the fall in chloride was masked by a concomitant rise in the plasma sodium; but relative to the sodium concentration, the fall in chloride was more or less commensurate with the rise in bicarbonate. Like the bicarbonate, the chloride returned to its pre-operative level within three or four days, if only one ureter was tied, or a sham operation performed. When both ureters were tied, the lowered chloride level was maintained throughout the period of anuria, falling still further as sulphate and phosphate accumulation became marked. The rise in sulphate and phosphate took place in the first instance at the expense of the plasma bicarbonate; only when very high concentrations of sulphate and phosphate were reached, was there a further fall in plasma chloride.

Sulphate. The rise in plasma sulphate was not rectilinear. For three or four days after both ureters had been tied, there was generally no marked change in the sulphate concentration. A rapid rise then occurred, which reached very high levels within a few days. In one case there was a rise from 2·9 to 27·3 milliequivalents per litre in five days. The level to which the sulphate concentration rose appeared to depend, at least in part, on how long the animal survived in the anuric state. The onset of diarrhoea did not inhibit the rise in sulphate; the two highest sulphate readings in this series were obtained in animals with severe diarrhoea.

Phosphate. The plasma phosphate concentration did not, in general, rise until some degree of sulphate accumulation was already present. The rise in phosphate was not as rapid, nor as marked as the rise in sulphate. The only animals in which no significant increase in phosphate occurred were those which died before the sulphate concentration reached high levels. The rise in phos-

phate was greatest in those cases in which sulphate accumulation was most marked.

Protein. As measured by the specific gravity of the plasma, there was, in all cases, a progressive rise in the plasma protein concentration during the period of anuria. In the protracted cases an increase of two to three gm. p.c. occurred. This increase was probably the result of the gross diminution in the plasma volume. However, the extent of the fall in plasma volume, as measured by the congo-red method, was somewhat greater than would be indicated by the rise in plasma protein. It is possible that there was actually some reduction in the total amount of protein present in the plasma.

Urea. Unlike sulphate and phosphate, the increase in plasma urea concentration was more or less rectilinear. There was considerable variation in the rate of increase, and no correlation could be found between the urea concentration and the time of death. The highest reading in the series was 643 mg. p.c.

Muscle analyses.

In assessing the significance of electrolyte estimations in muscle, certain features about the distribution of these electrolytes must be borne in mind. Although the extracellular water of muscle represents less than 20 p.c. of the total muscle water, by far the greater part of the muscle sodium, and even more of the muscle chloride, are to be found in this fraction. If, then, there is a gross diminution of the extracellular space of muscle, there should be a corresponding fall in the muscle sodium and the muscle chloride, provided no other change occurs. If the actual fall in muscle sodium and muscle chloride is less than the fall which might be calculated to occur as a result of diminution of the extracellular space, then sodium and chloride may be presumed to have entered the cells. If, despite marked diminution of the extracellular space, the muscle sodium and chloride concentrations remain constant, or rise, then it may be inferred that considerable amounts of these ions have moved into the cells. In the same way, if the muscle water concentration remains constant or rises, despite the fall in the extracellular space, it may be inferred that an increase in intracellular water has taken place. The potassium of muscle is, on the other hand, almost entirely confined to the cells. Diminution of the extracellular space would therefore result in an increase in the whole muscle potassium concentration provided no water moved into the cells. If, however, an increase in intracellular water did occur, the muscle potassium concentration would fall proportionally.

It is unfortunate that no accurate method exists for determining how much of the water in a given sample of muscle is extracellular, and how much intracellular. Until such a method is devised, the significance of electrolyte analyses in muscle can only be derived by inference from other estimations, such as the inulin space. This process of inference involves assumptions which make strictly quantitative assessment of the data impossible. In these experiments,

however, the changes observed were so gross, that a qualitative description of what occurred may none the less be made with some confidence.

Muscle water. Despite the marked fall in the extracellular fluid volume during the period of anuria, the whole muscle water concentration, in cases uncomplicated by diarrhoea, showed a rise of 2 to 7 p.c. This means that a considerable increase in the intracellular water must have occurred. In cases with prolonged diarrhoea the total muscle water was reduced.

Sodium. There was in all cases without diarrhoea an absolute increase in the muscle sodium. In view of the fall in extracellular space, this increase in muscle sodium concentration must represent a gross movement of sodium into the cells. When diarrhoea had been present for some time, the muscle sodium concentration fell.

Potassium. Changes in the muscle potassium concentration during the period of anuria were not marked. In two cases a significant fall in the muscle potassium did occur, but in both of these there was a considerable increase in the muscle water, despite the diminution of the extracellular space. Under these conditions it is possible that the fall in whole muscle potassium concentration is partly accounted for by a dilution of the intracellular contents, and does not actually represent a substantial loss of potassium from the cells.

Chloride. If the gross fall in the extracellular fluid volume is taken into account, and the fact that in the extracellular fluid the chloride concentration itself fell, then there is a clear indication in most cases that a substantial increase in intracellular chloride must have occurred. In some animals the increase in intracellular chloride did not appear to be as great as the increase in intracellular sodium. In view of the fall in chloride relative to sodium in the extracellular fluid this finding is difficult to explain. It is possible that in these cases sodium entered the extracellular fluid from the gastro-intestinal tract in greater amounts than chloride;[2] and since samples for analysis were only taken from one particular muscle at any one time, the possibility also exists that quantitatively different movements of sodium and chloride might have occurred in other organs. It must, moreover, be remembered that since the sodium concentration of the extracellular fluid is considerably higher than the chloride concentration, a fall in chloride relative to sodium may occur in the extracellular fluid, even though the absolute amount of sodium which enters the cells is greater than the absolute amount of chloride. Diarrhoea, while producing a marked fall in muscle sodium, had little effect on the muscle chloride.

Red blood cell analyses.

For the most part changes in the electrolyte composition of the red blood cells were not very significant. There was a slight fall in the chloride concentra-

[2] It has been observed that the sheep, being a ruminant, normally has large amounts of sodium in its digestive tract.

tion in all cases, and in the sodium concentration in cases with prolonged diarrhoea. The potassium remained more or less constant throughout.

Urine analyses.

Analysis of the urine in the renal pelvis was carried out primarily in order to investigate whether, as suggested by Atchley and Benedict (1927), the fall in plasma chloride concentration might not be due to accumulation of that ion in the residual pelvic urine. No such accumulation was found. It is interesting to note also, that after a period of three days' anuria the chloride concentration of the urine which had escaped from the polythene tube for several hours, was only three milliequivalents per litre. The sodium concentration of this urine was 48 milliequivalents per litre.

Morbid anatomy. [3]

There was a moderate amount of free fluid in the peritoneal cavity in all cases. The ureters, proximal to the ligatures, and the renal pelves were greatly distended, but the condition of the kidneys varied. In animals which died relatively quickly after operation, the kidneys were enlarged and turgid; in those with a longer survival time they were soft and necrotic although still somewhat enlarged. The liver and bowel appeared normal. In some cases the lungs also appeared normal, but in the majority they were more solid than usual, and obviously oedematous. There was, however, no fluid in the pleural cavity. In four animals gross haemorrhages were found in one or both adrenals.

Apart from the destruction of the renal tissue, the only constant changes found on microscopical examination were those in the lungs and those in the adrenals.

Lung. There was a generalized infiltration of the lung parenchyma with cells of the mononuclear type, and a marked swelling of the alveolar walls. Superimposed on this generalized change there were areas where the cellular infiltration was more dense, and where exudation had occurred into the alveoli. Patchy collapse was also a common feature (Figs. 1 and 2). Since the lungs were fixed within a few minutes of death, these processes must be regarded as having occurred ante mortem.

Adrenal gland. The changes found in the adrenals fall into four categories: hyperplasia of the cortex with formation of discrete vesicle-like spaces between the cords of cortical cells (Fig. 3); hyperplasia with marked distention of the vessels (Fig. 4); localized haemorrhages into the congested parenchyma (Fig. 5); gross haemorrhage completely disorganizing the architecture of the gland (Fig. 6). It is possible that these changes represent successive stages of the one pathological process.

[3] The histological examination was carried out with the help of Dr. John Forbes, of the Department of Pathology in the University of Melbourne.

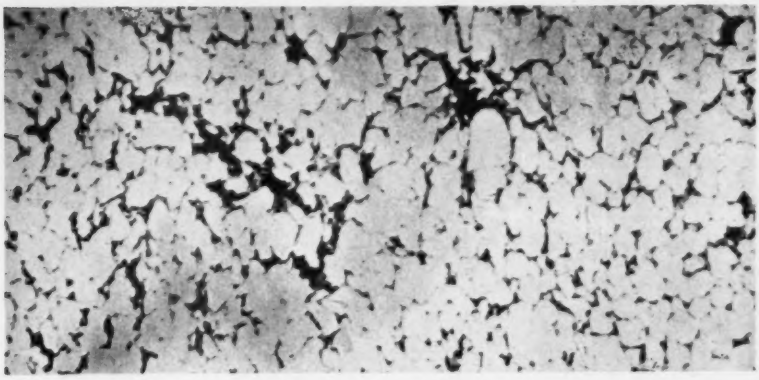

Fig. 1. Normal lung.

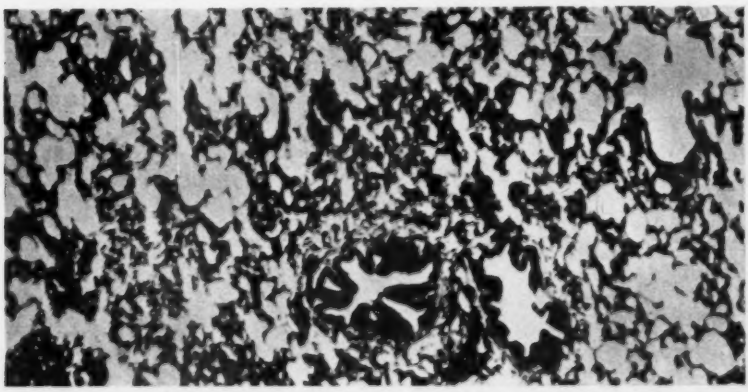

Fig. 2. Lung showing thickening of alveolar walls, patchy collapse and cellular infiltration.

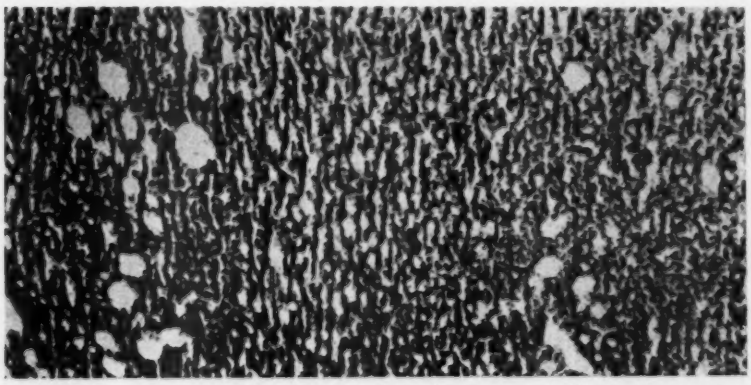

Fig. 3. Adrenal gland showing formation of vesicle-like spaces in the cortex.

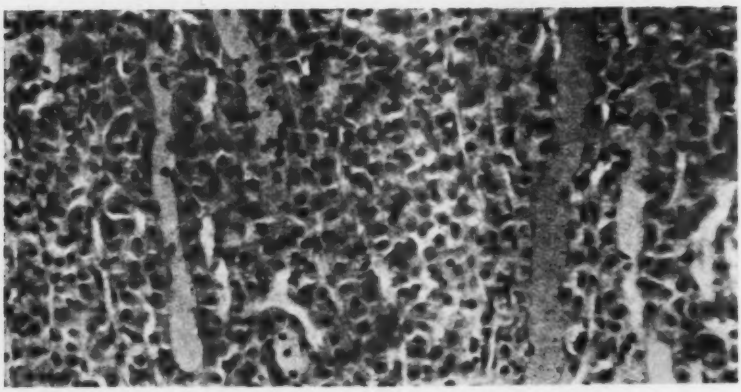

Fig. 4. Adrenal gland showing gross distention of cortical vessels.

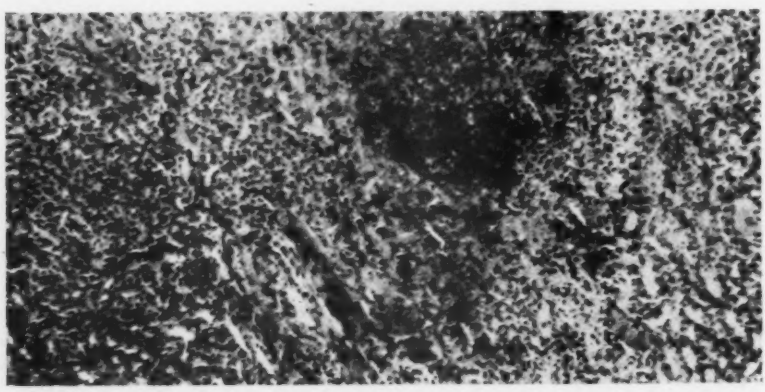

Fig. 5. Adrenal gland showing distention of vessels and small haemorrhages in the cortex.

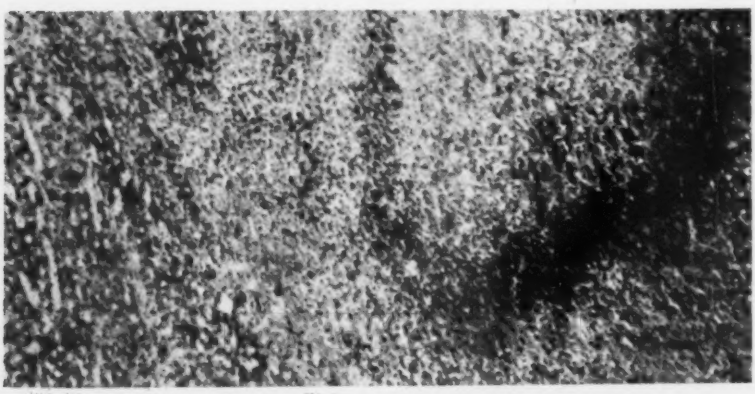

Fig. 6. Adrenal gland showing gross haemorrhage in the cortex.

DISCUSSION.

The main changes in the extracellular fluid in experimental anuria fall naturally into two groups: those associated with movement of water and electrolytes into the cells, and those resulting from the accumulation of metabolic end-products. The redistribution of water and electrolytes is not peculiar to anuria. The fall in plasma chloride and the concomitant rise in plasma bicarbonate occur when one ureter only is tied, or when a sham operation is carried out; and the change takes place within two or three hours of operation. The increase in intracellular chloride, sodium, and water also occurs when only one ureter is tied, and within a short time of operation. It is not improbable that the intracellular and the extracellular changes represent two aspects of the same phenomenon, and that the post-operative alteration in extracellular electrolyte pattern is due to the movement of electrolytes and water into cells. The precise significance of this phenomenon is not yet clear, but it appears to be a more or less general reaction to injury. A fall in plasma chloride concentration has been reported after surgical procedures in humans, and it frequently occurs in clinical anuria as soon as renal damage is manifest. There is no real indication in these experiments of the means by which this rapid alteration in electrolyte pattern is brought about. The gross changes found post-mortem in the adrenal gland do raise the possibility that adrenal hormones might in some way be implicated; but it is also possible that the morphological changes are the result of the physiological disturbance, rather than its cause. In this connection it is interesting to note that a fall in plasma chloride has been produced in rats by the administration of desoxycorticosterone acetate (Ralli, 1949).

In the presence of anuria the altered distribution of water and electrolytes persists. The changes resulting from the accumulation of metabolic end-products are thus superimposed upon an extracellular electrolyte pattern which has already been modified. This accumulation of end-products is represented for the most part by a progressive rise in plasma sulphate, phosphate, magnesium and non-protein nitrogen. A significant rise in plasma potassium is not an invariable finding either in the experimental or in human anuria, and there is little doubt that the marked rise which sometimes does occur in this condition is not to be explained simply by a failure to excrete the potassium liberated from cells. The rise in sulphate and phosphate, occurring first at the expense of bicarbonate, and later also at the expense of chloride, is associated with a fall of pH. In the sheep this fall of pH is only slight in cases uncomplicated by diarrhoea; but recent studies of anuria in the rat show that in this animal the pH falls regularly to 7·0 or less (Coats, 1951). The extent of pH fall in human anuria is variable, but appears to depend, at least in part, on factors other than the accumulation of sulphate and phosphate. An increase in the plasma magnesium concentration is a constant finding, but it does not reach levels at which pharmacological effects might be expected.

There is no clear indication in these investigations of the essential cause of death in anuria. The pH fall was slight, the rise in potassium inconstant, and not associated, in the two cases tested, with characteristic electrocardiographic changes; and there was no correlation between the time of death and the degree of sulphate, phosphate or urea accumulation. The combination of all these factors persisting for several days, and associated with alteration of the intracellular electrolyte pattern, might, in itself, be sufficient to explain death; but the possibility still exists that some single factor, which has not been investigated, may be more directly responsible. The pathological changes found post-mortem in the adrenal gland give grounds for believing that this organ contributes in some way to the disease process, but the physiological significance of the adrenal changes remains, for the time being, a matter of speculation.

SUMMARY.

Anuria was produced in the sheep by tying both ureters, and the alterations in the extracellular electrolyte pattern were studied daily.

A few hours after operation, a fall in extracellular chloride and a rise in extracellular bicarbonate occurred. This change appeared to be associated with movement of chloride, sodium, and water into cells. It could be produced by sham operation alone, but was maintained only if anuria was present.

The increase in plasma sulphate and phosphate concentration occurred at a later stage, and resulted in a gradual fall in bicarbonate and pH. When this increase was marked, a further fall of chloride also occurred.

In the sheep the fall in pH was slight, except when the condition was complicated by prolonged diarrhoea.

During the period of anuria, the extracellular fluid volume was greatly reduced, but despite this, there was, in the uncomplicated case, an increase in the water content of whole muscle.

A significant rise in plasma potassium concentration was not a constant finding, but a rise in plasma magnesium and a fall in plasma calcium occurred in all cases.

Of the changes studied in this investigation, no single one appeared sufficient, in itself, to account for the death of the animal. There was no correlation between the time of death and the rise in plasma sulphate, phosphate, potassium, magnesium or urea.

Gross pathological changes were found post-mortem in the adrenal gland.

Acknowledgments. It is a pleasure to record our thanks to Professor R. D. Wright for his helpful criticism of the manuscript; to Mrs. John Simon and Mr. J. A. Munro for their technical assistance; to Dr. D. A. Denton for his continued interest in the investigation; and to Mr. E. M. Hughes, Jun., for help in many directions.

TABLE 1.

Both ureters were clamped and tied on 9/4/51. Diarrhoea began 4 p.m. 13/4/51 and the animal was killed at 8 p.m.

Date	9·4 Pre-op.	9·4 Post-op.	10·4	11·4	12·4	13·4	13·4 8 p.m.
Weight (kg.)	40·3						34·5
Haematocrit (p.c.)	44	43	46	42	39	36	35
Blood pH	7·30	7·38	7·38	7·37	7·35	7·34	7·34
Plasma volume (ml.)	2,880					2,270	
Inulin space (litres)			12			8	
Plasma Na. (m.eq./l.)	137	137	143	139	136	138	137
Plasma K (m.eq./l.)	3·9	3·3	3·8	5·1	4·9	4·6	4·8
Plasma Ca (m.eq./l.)	6·17	5·55	5·71	5·58	5·40		4·77
Plasma Mg (m.eq./l.)	1·70	1·75	1·90	2·68	2·75	3·30	3·30
Plasma HCO_3 (m.eq./l.)	25·3	30·6	35·2	33·6	28·3	27·7	28·2
Plasma Cl (m.eq./l.)	108	103	101	97	97	97	97
Plasma SO_4 (m.eq./l.)	3·0	3·0	4·2	3·7	4·1	7·4	
Plasma PO_4 (m.eq./l.)	2·0	2·3	3·3	3·3	3·7	3·6	
Plasma protein (gm. p.c.)	6·7	6·2	6·1	6·3	7·1	7·9	7·3
Plasma urea (mg. p.c.)	18	21	54	111	172	235	298
Muscle water (p.c.)	76·7						80·3
Muscle Na (m.eq./kg.)	27						46
Muscle K (m.eq./kg)	78						68
Muscle Cl (m.eq./kg.)	19						28

TABLE 2.

Both ureters were clamped and tied on 12/2/51. The sheep did not develop diarrhoea until 20/2/51. On the morning of 21/2/51 the diarrhoea became melaena, and the sheep died at 5 p.m.

Date	12·2 Pre-op.	13·2	14·2	15·2	16·2	17·2	18·2	19·2	20·2	21·2	21·2 4.30 p.m.
Haematocrit (p.c.)	41	43	39	34	33	29	34	31	32	30	
Blood pH	7·48	7·50	7·47	7·49	7·41	7·40	7·40	7·47	7·41	7·43	7·43
Plasma Na (m.eq./l.)	150	149	152	151	150	150	148	154	149	148	141
Plasma K (m.eq./l.)	4·4	5·0	4·7	5·8	6·0	5·9	5·3	5·6	5·7	7·1	8·0
Plasma Ca (m.eq./l)	6·07	4·85	4·90	4·78	5·02	4·97	5·10	4·94	4·66	4·32	4·03
Plasma Mg (m.eq./l.)	2·50	3·00	3·30	4·30	4·42	4·43	4·66	4·17	4·50	4·40	5·50
Plasma HCO_3 (m.eq./l.)	21·2	34·8	32·9	34·1	29·9	30·4	25·2	28·3	25·2	25·0	22·3
Plasma Cl (m.eq./l.)	113	102	102	102	101	101	101	101	101	97	95
Plasma SO_4 (m.eq./l.)	3·6	4·7	3·9	4·1	7·0		7·0	12·0	15·0	16·5	
Plasma PO_4 (m.eq./l.)	3·4	3·0	3·4	3·4	3·3				5·6		
Plasma protein gm. p.c.)	6·8	6·4	6·4	6·5	6·8					8·2	
Plasma urea (mg. p.c.)		75		242				542			

TABLE 3.

A sham operation was carried out on 14/5/51 in which the right kidney was exposed and handled, but in which the ureter was not tied. Both ureters were tied at a subsequent operation on 16/5/51. The animal died on 20/5/51 without significant diarrhoea.

Date	14·5 Pre-op.	15·5	16·5 Pre-op.	17·5	18·5	19·5	20·5
Weight (kg.)	42·0						37·6
Haematocrit (p.c.)	48	44	47	43	43	43	46
Blood pH	7·25	7·29	7·20	7·37	7·37	7·35	7·34
Plasma volume (ml.)	2,030				860		
Inulin space (litres)			12·4		8·1		
Plasma Na (m.eq./l)	150	150	152	155	154	156	154
Plasma K (m.eq./l.)	3·7	3·8	3·8	4·0	4·1	4·1	4·0
Plasma Ca (m.eq./l.)	4·37	4·72	4·29	4·35	4·39	4·99	4·41
Plasma Mg. (m.eq./l.)	1·46	1·35	1·01	0·75	0·92	0·83	0·91
Plasma HCO_3 (m.eq./l.)	22·2	24·8	24·8	32·8	29·2	29·6	27·4
Plasma Cl (m.eq./l.)	109	108	112	108	106	102	102
Plasma SO_4 (m.eq./l.)	2·7	2·3	3·0	2·6	3·2	6·2	8·1
Plasma PO_4 (m.eq./l.)	3·8	3·2	3·4	2·7	4·0	4·5	3·7
Plasma protein (gm. p.c.)	6·8	6·9	6·9	7·0	7·3	7·1	7·3
Plasma urea (mg. p.c.)	26	29	41		111		288
Muscle water (p.c.)	74·5				76·6		
Muscle Na (m.eq./kg.)	35				41		
Muscle K (m.eq./kg.)	79				82		
Muscle Cl (m.eq./kg.)	32				28		
Red Cell Na (m.eq./l.)	106				107		
Red Cell K (m.eq./l.)	8				8		
Red Cell Cl (m.eq./l.)	65				54		

TABLE 4.

In this case a polythene tube was tied into the right ureter, and led to the exterior. This operation together with left nephrectomy was carried out on 2/4/51. The blood loss during operation was about 500 ml. By clamping the polythene tube anuria could be produced, but samples of the urine in the renal pelvis could be obtained at will. During the night of 5/4/51 the clamp became detached and an unknown amount of urine was passed. A specimen of the urine on the following day, 6/4/51, was analysed. Urine was allowed to flow freely from the polythene tube for three days, and the plasma electrolyte pattern was determined at the end of this period.

Date	2·4 Pre-op.	3·4	4·4	5·4	6·4	7·4	8·4	9·4
Weight (kg.)	50·5			48·5				
Haematocrit (p.c)	50	39	41·2	39				42
Blood pH	7·44	7·52	7·51	7·52				7·46
Plasma volume (ml.)	2,840		1,600					
Inulin space (litres)	13·1		10·3					
Plasma Na (m.eq./l.)	138	140	142	138				135
Plasma K (m.eq./l.)	4·0	4·7	4·6	5·0				3·6
Plasma Ca (m.eq./l.)	5·68	4·77	4·77	4·76				
Plasma Mg (m.eq./l.)	1·60	1·80	2·00	2·40				
Plasma HCO_3 (m.eq./l.)	24·3	34·3	33·8	37·4				22·2
Plasma Cl (m.eq./l.)	103	94	94	93				103
Plasma SO_4 (m.eq./l.)	3·0	3·1	2·0	2·0				4·2
Plasma PO_4 (m.eq./l.)	3·2	2·2	2·3	2·3				2·4
Plasma protein (gm. p.c.)	7·1	6·4	6·7	6·3				7·1
Plasma urea (mg. p.c.)	40	76	172	207				186
		10 a.m. 5 p.m.						
Urine Na (m.eq./l.)		95 86	67	41	48			
Urine K (m.eq./l.)		47 36	35	37	28			
Urine Cl (m.eq./l.)		15 26	41	12	3			
Urine NH_3 (m.eq./l.)		2·0 0·5	0	0	2·3			
Urine pH		7·20 7·24	6·30	6·15	6·80			

TABLE 5.

A sham operation was carried out on 30/5/51; the right ureter was tied at a second operation on 1/6/51, and the left ureter at a third operation on 4/6/51. Diarrhoea began on 7/6/51, and was marked at the time of death, which occurred at 8 p.m. on 8/6/51.

Date	30·5 Pre-op.	31·5	1·6 Pre-op.	2·6	3·6	4·6 Pre-op.	5·6	6·6	7·6	8·6
Weight (kg.)	40·1								39·0	
Haematocrit (p.c.)	44	43	43	41		40	41	41	41	40
Blood pH	7·29	7·43	7·33	7·43	7·40	7·31	7·45	7·36	7·35	7·35
Plasma volume (ml.)	2,680							1,880		
Plasma Na (m.eq./l.)	150	154	155	154	153	151	151	150	150	149
Plasma K (m.eq./l.)	3·9	4·3	4·2	4·4	4·5	4·2	4·1	4·6	4·8	3·5
Plasma Ca (m.eq./l.)	5·42	4·96	4·62	4·70	4·45	4·60	4·75	4·80	5·10	5·10
Plasma Mg (m.eq./l.)	1·12		1·75	1·68	1·73	1·40	1·45	1·63	1·47	2·02
Plasma HCO_3 (m.eq./l.)	22·1	29·0	27·9	30·2	28·4	24·3	30·7	27·5	28·2	24·2
Plasma Cl (m.eq./l.)	107	105	106	103	105	106	102	105	105	105
Plasma SO_4 (m.eq./l.)	2·5	1·9	2·1	2·4	6·9	8·3	8·6	8·7	8·5	9·7
Plasma PO_4 (m.eq./l.)	3·4	3·4	4·0	4·1	4·5	4·0	4·0	3·3	3·3	4·4
Plasma protein (gm. p.c.)	6·2	6·8	6·9	6·7	6·7	6·6	6·7	7·0	7·5	9·0
Plasma urea (mg. p.c.)	44	41	18	28	34	48	58	98	159	210
Muscle water (p.c.)	73·7			81·0						
Muscle Na (m.eq./kg.)	44			63						
Muscle K (m.eq./kg.)	75			70						
Muscle Cl (m.eq./kg.)	25			26						
Red cell Na (m.eq./l.)	110						104			
Red cell K (m.eq./l.)	9						7			
Red cell Cl (m.eq./l.)	68						61			

TABLE 6.

Both ureters were tied on 17/4/51. For the first three or four days this sheep drank almost a litre of water a day, which was about twice the usual amount under these circumstances. After the fourth day, however, the water intake dropped and remained less than half a litre a day until death. Diarrhoea began on 22/4/51 and during the following four days became exceedingly severe. The animal died at 4 p.m. on 26/4/51.

Date	17·4 Pre-op.	18·4	19·4	20·4	21·4	22·4	23·4	24·4	25·4	26·4
Weight (kg.)	36·6			34·7						30·9
Haematocrit (p.c.)	40	39	40	35		35	36		35	49
Blood pH	7·44	7·61	7·58	7·43	7·44	7·36	7·38			
Plasma volume (ml.)	2,210					2,200				1,450
Inulin space (litres)		9·0				8·3				2·6
Plasma Na (m.eq./l.)	136	139	139	136	140	139	148	149	150	148
Plasma K (m.eq./l.)	3·2	4·8	4·7	4·8	5·0	4·6	5·0	5·1	5·2	6·5
Plasma Ca (m.eq./l.)	5·17	4·83	4·75	4·74	4·74	4·62	4·53	3·91	3·67	3·65
Plasma Mg (m.eq./l.)	1·75	2·25	2·56	2·80	2·90	2·60	2·70	2·45	2·55	2·90
Plasma HCO_3 (m.eq./l.)	26·0	33·0	34·7	31·6	27·1	22·5	23·2	25·2	25·3	24·2
Plasma Cl (m.eq./l.)	103	100	97	98	99	100	97	97	94	90
Plasma SO_4 (m.eq./l.)	3·5	3·7	3·8	5·6		9·7	12·0	12·3	13·0	17·5
Plasma PO_4 (m.eq./l.)	2·9	2·6	2·2	3·4	3·4	3·8	5·2	4·7	6·0	5·9
Plasma protein (gm. p.c.)	7·5	7·0	7·4	7·8	8·0	8·3	8·4	8·0	8·7	9·9
Plasma urea (mg. p.c.)	21	141	183	236	271	440	420	493	643	
Muscle water (p.c.)	78·4									69·3
Muscle Na (m.eq./kg.)	37									30
Muscle K (m.eq./kg.)	77									77
Muscle Cl (m.eq./kg.)	26									26
Diarrhoeal fluid Na (m.eq./l.)										101
Diarrhoeal fluid K (m.eq./l.)										35
Diarrhoeal fluid Cl (m.eq./l.)										67

TABLE 7.

This sheep was shorn the day before operation, and was found during operation on 7/5/51 to have a haematocrit of 62 p.c. Since a haemotocrit of this order is frequently associated in sheep with pneumonia, only one ureter was tied, and the animal was given an intensive course of chemotherapy for several days. The haematocrit returned to normal in three days and was still normal on 14/5/51, when the second ureter was tied. Moderate diarrhoea began on 12/5/51 and continued with increasing severity until death, which occurred at 9 p.m. on 19/5/51.

Date	7·5 Pre-op.	8·5	10·5	14·5 Pre-op.	15·5	16·5	17·5	18·5	19·5
Weight (kg.)	50·8								41·4
Haematocrit (p.c.)	62		45	45	44	43	39	40	40
Blood (pH)	7·33	7·33	7·33	7·17	7·05	7·10	7·15	7·16	7·04
Plasma volume (ml.)	2,390			1,440					
Plasma Na (m.eq./l.)	152	151	153	148	146	150	146	139	141
Plasma K (m.eq./l.)	3·9	3·9	3·7	2·7	3·3	5·1	5·2	5·5	6·4
Plasma Ca (m.eq./l.)	5·00	4·97	5·27	4·65	5·28	4·88	4·01	3·19	3·49
Plasma Mg (m.eq./l.)	0·93	1·34	1·39	1·67	2·10	2·28	2·34	2·20	2·12
Plasma HCO_3 (m.eq./l.)	25·0	32·1	31·6	20·3	18·3	17·9	18·0	14·9	11·9
Plasma Cl (m.eq./l.)	108	102	98	105	106	103	98	83	87
Plasma SO_4 (m.eq./l.)	0·9	1·3	2·2	2·9	6·8	16·5	17·5	26·2	27·3
Plasma PO_4 (m.eq./l.)	3·4	2·9	2·5	3·5	5·1	7·2	9·4	10·5	10·8
Plasma protein (gm. p.c.)	7·5	7·3	7·3	9·3	8·7	8·8	8·4	8·6	8·3
Plasma urea (mg. p.c.)	22		40	129	254	362		575	
Muscle water (p.c.)	76·8					73·5			
Muscle Na (m.eq./kg.)	38					26			
Muscle K (m.eq./kg.)	85					82			
Muscle Cl (m.eq./kg.)	25					21			
Red Cell Na (m.eq./l.)	102					93			
Red Cell K (m.eq./l.)	11					12			
Red Cell Cl (m.eq./l.)	68					65			

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IMPAIRED RESPONSE OF THE ADRENAL MEDULLA FOLLOWING BARBITURATE ADMINISTRATION

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Much emphasis has been placed on the effect of barbiturate on the vagal mechanism. In this present paper we have investigated the effect of Nembutal (Na-ethyl-methyl butyl barbiturate) on the pressor response to acetylcholine which is mediated through the suprarenal medulla.

METHOD.

Cats were anaesthetized with ether and chloroform and then chloralose intravenously (8 ml. of 1 p.c. solution per kilo). The vagi were cut and the carotid blood pressure recorded. The femoral vein was cannulated for intravenous injections. Nembutal was used in a dose of 70 mg. and it was injected slowly intravenously. In certain instances the animal was atropinized. In one instance the left suprarenal gland was removed (under the same anaesthesia as above) and then extracted for assay with sand in saline and boiled.

EXPERIMENTAL.

1. *The effect of Nembutal on the vasodepressor effect of acetylcholine.*

Nembutal reduces the depressant effect of acetylcholine on the cat's blood pressure; e.g. before the administration of Nembutal to an anaesthetized cat with the vagi cut two injections of 4 μg. acetylcholine were given which resulted in a fall of blood pressure of 50 mm. Hg. on each occasion, the initial blood pressure being 120 mm. Hg. (Ac, Fig. 1). 70 mg. Nembutal were then given slowly intravenously and $3\frac{1}{2}$ minutes after the commencement of this injection the blood pressure fell 14 mm. Hg. in response to 4 μg. acetylcholine and $2\frac{1}{2}$ minutes later the blood pressure fell 16 mm. Hg. in response to the same dose (Ac. Fig. 1).

2. *Response of the blood pressure to adrenaline before and after the administration of Nembutal.*

With an initial blood pressure of 188 mm. Hg. in a cat with the vagi cut, the response to the injection of 20 μg. adrenalin was a rise in blood pressure of 20 mm. Hg. (Fig. 2). The blood pressure slowly declined to 124 mm. Hg. and

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the injection of 20 μg. adrenaline resulted in a rise in blood pressure of 22 mm. Hg. When the blood pressure fell to 136 mm. Hg. the injection was repeated and the blood pressure rose 42 mm. Hg., i.e. the response was more marked with a lower initial blood pressure. Following the injection of 70 mg. Nembutal intravenously, the response to adrenaline, after some recovery of blood pressure had occurred (right hand panel, Fig. 2) was even greater.

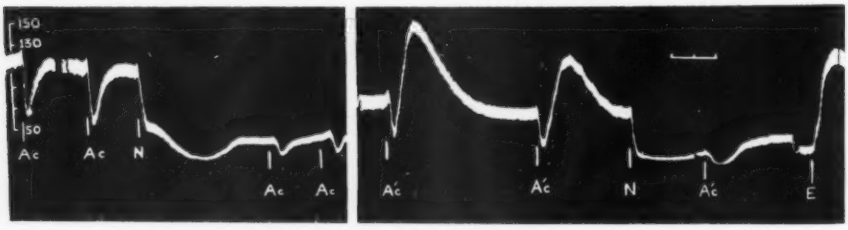

Fig. 1.

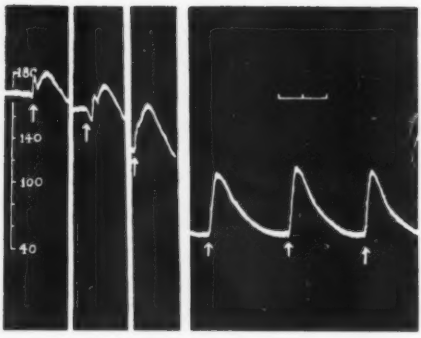

Fig. 2.

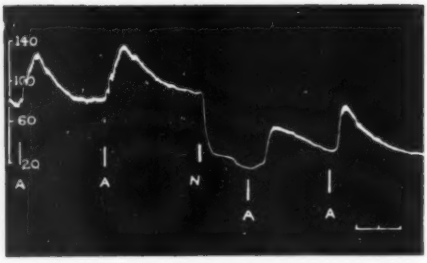

Fig. 3.

Fig. 1. Response of the blood pressure of an atropinized cat under ether and chloralose anaesthesia to acetylcholine before and after Nembutal. Between the panels 3 mg. atropine sulphate injected after interval of 45 min. (to allow recovery from the previous effects of Nembutal). At Ac 4 μg. acetylcholine, at N 70 mg. Nembutal, at A'c 2 mg. acetylcholine and at E 1/10 extract of left suprarenal gland injected. Blood pressure in mm. Hg. Time in minutes. Details in text.

Fig. 2. Response of the blood pressure of a vagotomized cat under ether and chloralose anaesthesia to adrenaline before and after Nembutal. At the arrows 20 μg. adrenaline injected. Between the third and fourth panel 70 mg. Nembutal injected. Interval between Nembutal injection and first adrenaline injection (fourth panel) 4 minutes. Blood pressure in mm. Hg. Time in minutes. Details in text.

Fig. 3. Responses of the blood pressure of a vagotomized atropinized cat under ether and chloralose anaesthesia to adrenaline before and after the injection of Nembutal. At A response to 20μg. adrenaline. At N 70 mg. Nembutal injected. Blood pressure in mm. Hg. Time in minutes. Details in text.

Following the injection of 70 mg. Nembutal intravenously, when the animal is in a shocked state with a low blood pressure, there is temporarily some impairment in the response to adrenaline, mainly shown by a delay but the magnitude of the response is little impaired within two minutes of the injection. In one experiment with an atropinized animal there was a rise of 55 mm. Hg. in the blood pressure (A, Fig. 3) following the injection of 20 μg. adrenaline, which was repeated. Two minutes after the injection of 70 mg. Nembutal (N) the blood pressure showed a delayed rise (A, Fig. 3) from the near zero level of 8 mm. Hg. of the same order as following the first injections. A further injection of adrenaline produced a response without delay (A, Fig. 3).

3. *Reduction of pressor response (nicotine effect) of acetylcholine.*

The pressor response to acetylcholine is impaired greatly by Nembutal. In one experiment 2 mg. acetylcholine were injected into an atropinized cat when the blood pressure of 60 mm. Hg. rose 85 mm. Hg. after an initial slight fall. 4½ minutes after the administration of 70 mg. Nembutal intravenously there was a more marked preliminary fall in blood pressure which then rose 20 mm. Hg. These findings are illustrated in Fig. 4, where at Ac, 2 mg. acetylcholine, and at N 70 mg. Nembutal were injected. In a further experiment (see Fig. 1) where the initial blood pressure was 85 mm. Hg. the response to 2 mg. acetylcholine (at A′c) was an overall rise of 70 mm. Hg. An injection of 2 mg. acetylcholine given 6½ minutes later (at A′c) resulted in a rise of 57 mm. Hg. In both cases there was an initial depressor effect. 70 mg. Nembutal were then administered slowly (at N) and 2½ minutes after the injection the blood pressure was 25 mm. Hg. When 2 mg. acetylcholine were injected (at A′c) only a recovery of blood pressure was recorded following an initial slight slow fall. The left suprarenal gland was then removed and a saline extract made. Following the injection of 1 ml. of this extract 30 minutes later, containing one-tenth of the gland, there was a rise in blood pressure of 85 mm. Hg. Thus the failure of response of the blood pressure was not due to complete removal of adrenaline from the gland by the previous injections.

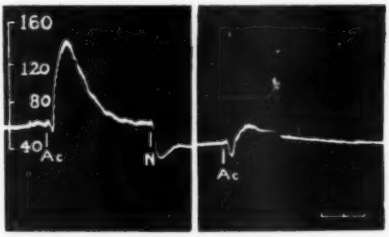

Fig. 4. Responses of the blood pressure of a vagotomized atropinized cat under ether and chloralose anesthesia, to acetylcholine before and after injection of Nembutal. At Ac response to 2 mg. acetylcholine. At N 70 mg. Nembutal injected intravenously. Interval between panels two minutes. Blood pressure in mm. Hg. Time in minutes. Details in text.

It is therefore evident that the pressor response to acetylcholine is considerably reduced following the injection of Nembutal. This might be due to

depression of the response of the ganglion cell to acetylcholine stimulation or depression of the output of adrenaline by the ganglion cell or depression of responsiveness of the sympathetic endings to adrenaline. If the responsiveness of the blood pressure to adrenaline were impaired the injection of adrenaline after Nembutal should produce a smaller rise in the blood pressure than before Nembutal. There was a temporary depression and delay of response to adrenaline during the extreme hypotensive stage following Nembutal administration but within two minutes of injection even though there was still a delay in the response the magnitude of the response was as great as that before the injection of Nembutal. Further at the stage when the response of the blood pressure to a large dose of acetylcholine was greatly depressed the response to adrenaline was practically normal. It is therefore, concluded that the reduced pressor response to acetylcholine is due to impaired sensitivity of the ganglion cell to acetylcholine stimulation or decreased output of adrenaline.

In addition there is impairment of the fall of pressure to small doses of acetylcholine. In the experiment recorded (Fig. 1) this may be partly accounted for by the initial carotid pressure being higher before Nembutal administration than after.

DISCUSSION.

It has already been shown that stimulation of the vagus after the injection of sodium amytal intravenously produces an impaired response (Lieb and Mulinos, 1929). De Waele (1925) found that barbiturate affected the peripheral part of the inhibitory mechanism of the heart. Shafer, Underwood and Gaynor (1930) confirmed the findings of Lieb and Mulinos. Wright, Florey and Jennings (1938) showed that barbiturates caused inhibition of movement and of secretion in the colon following pelvic nerve stimulation. Wright, Jennings, Florey and Lium (1940) further showed that some anaesthetics reduce secretory activity produced by nerve stimulation. Trethewie (1942) showed that acetylcholine was liberated from the bowel following nerve stimulation, and that this output was inhibited following perfusion with sodium amytal. We did not determine whether this inhibition occurred at the preganglionic or postganglionic level in the intestine. In the present paper we have shown that the sympathetic ganglion of the autonomic system is similarly affected by barbiturate as expressed in the suprarenal medulla. Other sympathetic ganglia may be similarly affected. It is possible that some of the favourable effects of barbiturate therapy in hyperpiesis may be due to this mechanism. It is to be noted that more recent sympathetic-acting drugs, e.g. tetraethylammonium, have been employed in the treatment of hypertension, though they have not superseded the barbiturates.

SUMMARY.

The depressor response of the cat blood pressure to acetylcholine is reduced following the injection of Nembutal intravenously.

The pressor response to large doses of acetylcholine in the atropinized animal is very greatly reduced by the injection of Nembutal intravenously—at a stage when the pressor response to adrenalin is practically unchanged.

The significance of the above findings is discussed.

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THE RESPONSE OF THE MACROPHAGE TO ANTIGENIC AZOPROTEIN, STUDIED IN THE RABBIT EAR CHAMBER PREPARATION

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Since the well-known work of Sabin (1939) there has been increasing interest in the cytology of immunity. Using R-salt-azoprotein as the antigen, Sabin observed in fixed sections of rabbit lymph nodes and in cell suspensions appearances which she interpreted as representing the shedding of peripheral portions of the cytoplasm of the macrophages which had ingested the coloured antigen. She suggested that the shed fragments contained the antibody. Following these earlier studies interest then shifted to the possible rôle of the lymphocyte (Ehrich and Harris, 1942; Dougherty and White, 1943, 1944, 1945, 1947; Dougherty, Chase and White, 1944, 1945a, 1945b; Dougherty, White and Chase, 1944; White and Dougherty, 1944, 1945a, 1945b, 1946; White, 1946; Harris, Grimm, Mertens and Ehrich, 1945; Thatcher, Haughton and Ziegler, 1948; Valentine, Craddock and Lawrence, 1948; Harris and Harris, 1948, 1949; Craddock, Valentine and Lawrence, 1949; Spear, 1948; Habel, Endicott, Bell and Spear, 1949) and to that of the plasma cell (Gormsen, 1941; Bjorneboe and Gormsen, 1941, 1943; Bjorneboe, Gormsen and Lundquist, 1947; Fagraeus, 1947, 1948a, 1948b; Ehrich, Drabkin and Forman, 1949; Forman, Drabkin and Ehrich, 1949). Ehrich, Harris and Mertens (1946) were not able to demonstrate significant amounts of antibody in an extract of cells consisting mainly of macrophages, during the period of maximum antibody formation. Harris and Ehrich (1946) however, provided some experimental evidence on the rôle of the macrophage in a report that after injection of particulate antigen into the pad of the rabbit's foot, soluble products which yet retained the specificity of the antigen (in that they would react with the antibody *in vitro*) could be recovered from the lymph from the popliteal node.

There is now much evidence that the macrophage is concerned in at least the primary ingestion of even soluble antigens, whatever its subsequent rôle in the elaboration of the antibody may be (Burnet and Fenner, 1949). More recent studies using coloured antigenic azoproteins have served to confirm that these too are engulfed by the macrophage (Kruse and McMaster, 1949; Gitlin, 1950; Latta, Gitlin and Janeway, 1951). The last-mentioned studies all involved the

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examination of histological sections of the tissues of the animal for the presence of the coloured antigen. The elegant investigations of Coons and his co-workers (Coons *et al.*, 1942; Coons and Kaplan, 1950; Coons *et al.*, 1950; Kaplan, Coons and Deane, 1950; Hill, Deane and Coons, 1950; Coons, Leduc and Kaplan, 1951), in which a fluorescent antibody has been employed as a sensitive and specific histochemical stain to locate the presence of antigen have also involved the use of sections mounted on slides.

The rabbit ear chamber preparation, however, is a very suitable one for the examination of the behaviour of individual cells over extended periods of time. It was felt, therefore, that a study of the changes in the individual macrophage during and subsequent to the uptake of coloured antigenic azoprotein would be worthwhile, as affording in some ways a better impression of the behaviour of the cell than the study of a histological section, which can only show the process arrested at a single instant, and is subject to technical artefacts.

Experimental.

The techniques used in this work have been described in a previous paper (Robertson, 1951). Two antigens were employed, the R-salt-azoprotein used by Sabin (1939) and prepared according to the directions of Heidelberger, Kendall and Soo-Hoo (1933), and Evans Blue (T 1824) linked to horse serum according to the method of Kruse and McMaster (1949). All the rabbits were non-immune animals.

It was found that the R-salt complex was not intensely enough coloured to be readily detectable in the film of living tissue, particularly as its colour in the cell is rather similar to that of haemoglobin. The observations here reported therefore have been made using the Evans blue azoprotein.

The access pin was removed from the ear chamber by the method previously described (Robertson, 1951), and the antigen injected using a specially made brass-tipped perspex nozzle (Fig. 1), carried, like the pin vice, in the rotating nosepiece of the microscope. An "Agla" micrometer syringe and a flexible tube filled with mercury complete the injection assembly. 50,000 units of crystalline penicillin and 0·075 gm. of streptomycin were given intravenously in a vein in the other ear just before the injection of the antigen.

Thirteen animals bearing chambers were used in this work. Antigenic azoprotein was successfully introduced into four of these, without causing undue trauma or sepsis. Two injections were made into one of the animals, first of the R-salt complex, and later, after resting for six weeks so that no visible trace of the antigen remained of the Evans Blue complex. Unlinked Evans Blue (0·75 p.c. in 0·9 p.c. saline) was injected into two animals, methylene blue (1:400 in 0·9 p.c. saline) was introduced via the local venous system in one, and Evans Blue azoprotein in a similar fashion in another.

OBSERVATIONS.

The methylene blue solution was used to make some preliminary observations. The solution was introduced into an ear vein distal to the chamber, and was held in the ear by compression of the vessels at the base. After two minutes the digital compression was released and the solution allowed to enter the general circulation. The film of tissue, particularly the region of the veins,

became stained markedly blue. The animal was immediately prepared for microscopic observation, and the first observations were made approximately seven minutes after introduction of the solution. At this time the local venous circulation was seen to be arrested and adventitial macrophages with all their processes were intensely stained (Fig. 2). Staining of nerve fibres began after about fifteen minutes and continued till about 45 minutes. The macrophages presented a striking appearance, and the shedding of peripheral portions of the cytoplasmic processes was clearly observed at ten minutes, as can be seen from the Figure. This phenomenon can thus apparently occur within a very few minutes of the ingestion of the dye by the cell. It was long ago studied in macrophages by Ranvier (1900) and was in fact the property which led him to call these cells "clasmatocytes."

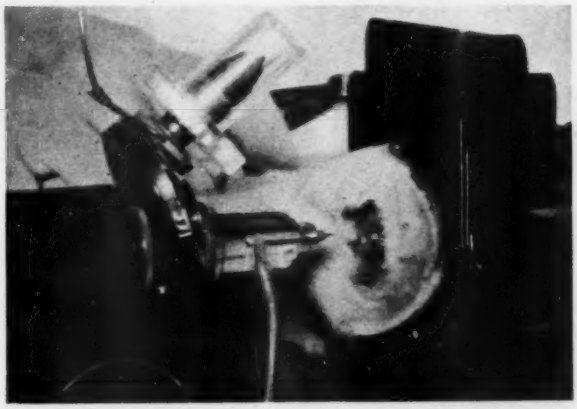

Fig. 1. Injection nozzle and pin vice (the latter with perspex cap in place) mounted in revolving nosepiece of the microscope.

In order to bring it quickly into contact with the tissue macrophages, the blue antigenic azoprotein was introduced in other chambers through the access pinhole, and not via the local venous system. Slow injection ensured that the solution flowed over the film of tissue without disrupting it, passing between the tissue and the coverslip, or between the tissue and the top surface of the table, depending on whether the chamber had its access pins in the base or in the lid. With the naked eye the solution could be seen to be occupying irregular areas of the field. The process was watched from below during the actual injection, by means of the inverted modification of the microscope previously described (Robertson, 1951). It could then be seen that the dye complex became fixed within a few moments to the tissue components.

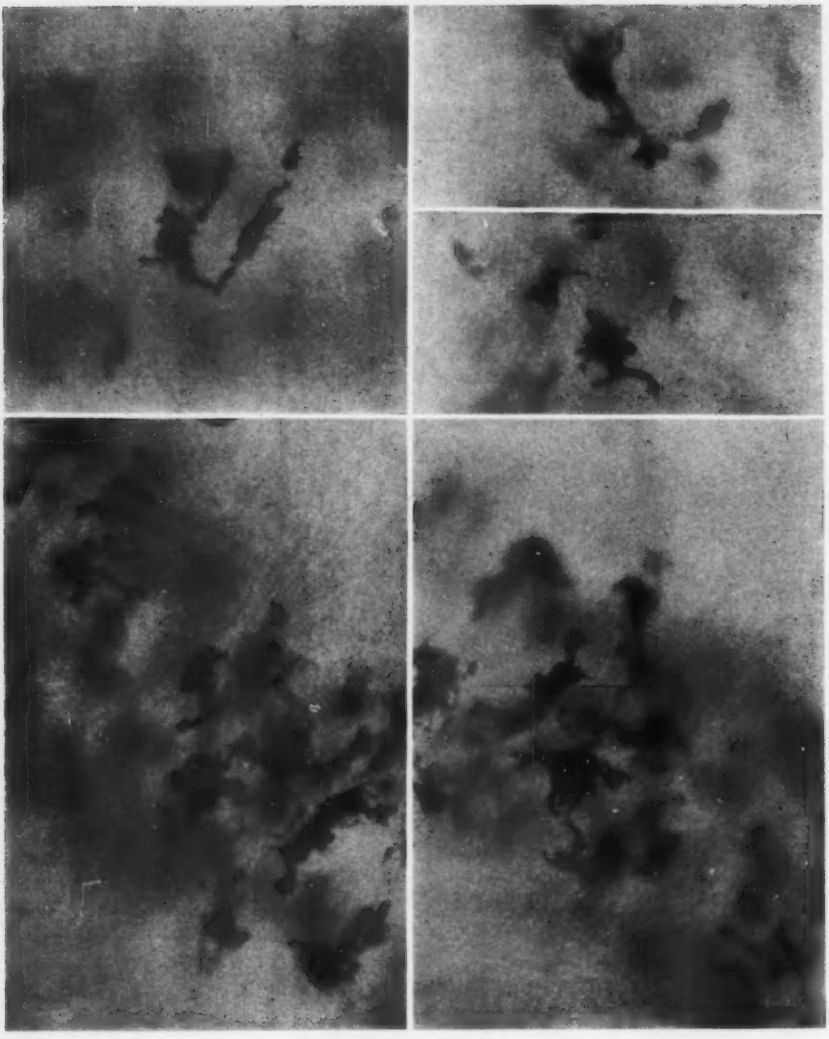

Fig. 2. Adventitial macrophages vitally stained with methylene blue. The shedding of cytoplasmic processes is visible.

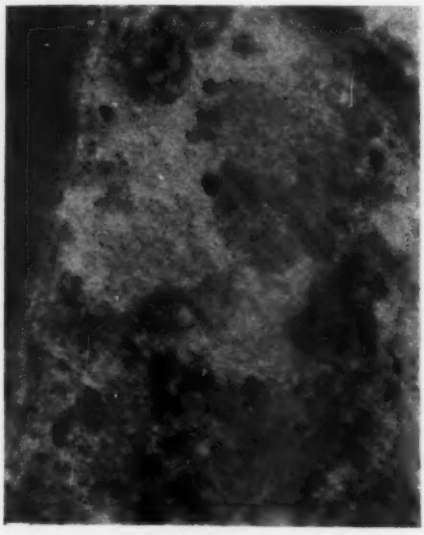

Fig. 3.

Fig. 4.

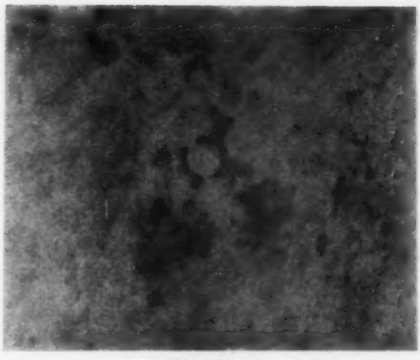

Fig. 5.

Fig. 6.

Fig. 3. Field 2 hours after injection of dye-protein complex. Staining of small lymphoid cells is shown.

Fig. 4. 4½ hours after injection of dye-protein complex. Two macrophages are visible, with an appearance suggesting the shedding of a peripheral portion of cytoplasm from one cell.

Fig. 5. 6½ hours after injection of dye-protein complex. Two macrophages are shown, the lower cell disintegrating. A cell resembling a large lymphocyte is shown in the upper portion of the figure, with dye-complex present in its cytoplasm.

Fig. 6. 10 hours after injection of dye-protein complex. Two macrophages with granular cytoplasm are shown.

Fig. 7.

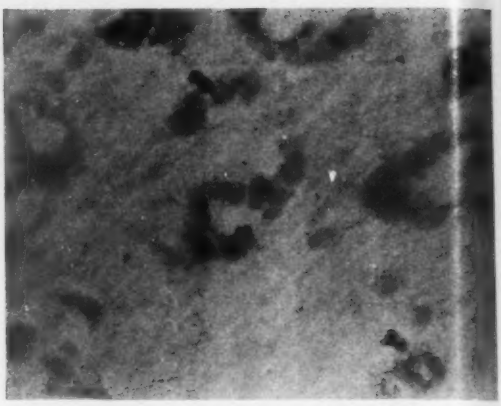

Fig. 8.

Fig. 9.

Fig. 7. 5th day after injection. A group of macrophages with unstained nuclei. Three oval deeply stained bodies are visible on the periphery of the group.

Fig. 8. 5th day. Macrophages with unstained nuclei and granular and disintegrating cytoplasm. An oval deeply stained body in the upper right hand corner shows a fragment of cytoplasm applied to its surface.

Fig. 9. 5th day. A disintegrating macrophage.

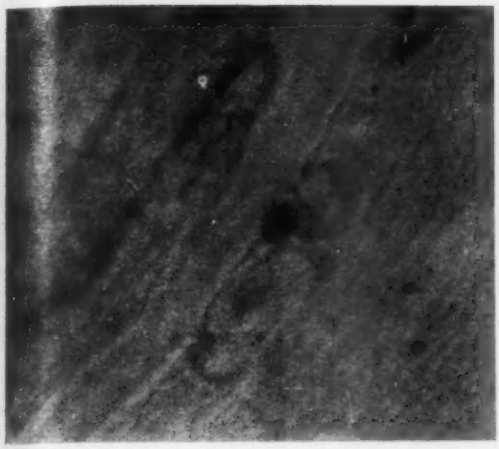

Fig. 10.

Fig. 11.

Fig. 12.

Fig. 10. 7th day. A group of cells near a small capillary vessel, including an oval deeply stained body.

Fig. 11. 8th day. The same group as Fig. 10, showing loss of dye-complex from the granular macrophages and persistence of the oval body.

Fig. 12. 9th day. The same group as Figs. 10 and 11. Only the oval body remains, and it has moved to the other side of the vessel.

No early "vital staining" of the macrophages was seen after the injection as was the case with the plain methylene blue solution. At two hours irregular granular masses of pigment were visible, and there was definite staining of small rounded cells resembling lymphocytes (Fig. 3). After approximately four hours the macrophages were faintly visible. They did not assume the very highly branched forms shown in Fig. 2, but some less branched forms were observed (Fig. 4). Although the cells were faint and difficult to photograph, evidence of the shedding of portions of cytoplasmic processes is provided by Fig. 4. At 6½ hours after the injection the macrophages were more definitely visible, showing a clear oval central area representing the nucleus, surrounded by an usually diffusely blue-staining cytoplasm. However, some cells (Fig. 5) were highly granular and apparently disintegrating. Staining of the cytoplasm of a smaller cell resembling a large lymphocyte is also seen in the upper part of Fig. 5. At 10 hours (Fig. 6) the cells were stained much more deeply, and the cytoplasm was almost invariably granular. Staining of small cells resembling lymphoid cells was much less evident.

Fig. 13.

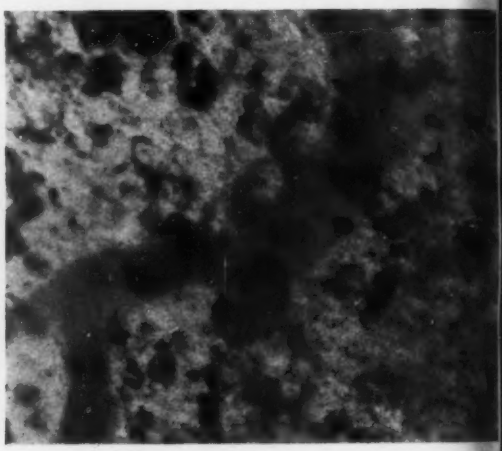

Fig. 14.

Fig. 13. 6th day. A deeply stained oval body, with two cytoplasmic fragments applied to its surface.
Fig. 14. 18th day after injection of unlinked dye. Many cells containing blue granules are still present.

At 24 hours, and still more at 48 hours, groups of macrophages staining intensely blue were very evident throughout the field. Most of the cells had a granular blue cytoplasm, although in some cells there were areas of cytoplasm in which the dye appeared to be distributed more diffusely. Non-nucleated blue cytoplasmic fragments were quite conspicuous lying among the groups of living and nucleated cells. At 51 hours, in one animal, a blue-stained macrophage was seen to divide by mitosis.

On succeeding days further evidence of the ingestion of the dye-complex by other groups of macrophages continued to appear. Many of the cells became more intensely coloured than hitherto, while the cytoplasm of most cells became rather more granular, containing blue-staining granules of very variable size and depth of colour. At the same time evidence of the shedding of peripheral fragments of cytoplasm continued to be most striking, together with complete disintegration of some of the cells (Figs. 8 and 9). Many cytoplasmic fragments were ingested by other neighbouring cells, producing remarkable "target" appearances.

On and after the fifth day relatively small (12–15μ) oval, comparatively immobile intensely coloured bodies appeared, usually in close proximity to larger cells which were obviously macrophages or fragments of macrophages. Two of these are shown in Fig. 7 and in Figs. 10 to 13. Several of them were observed for some days. In none of them was a clear nuclear zone visible. This suggested the possibility that the bodies were residual nuclei, which had taken up the dye after the death of the cell, and that the blue-staining oval granule visible in some (Figs. 7 and 10 to 12) might represent a nucleolus. A point against this view, however, was the fact that in one case (see Figs. 10 to 12) one of these bodies was observed to migrate from one side of a small capillary vessel to the other.

This latter body was first observed on the sixth day, and photographed on the seventh day (Fig. 10). It lay in close proximity to a ragged group of obvious adventitial macrophages—granular cells with clear oval unstained nuclei. On the next day (eighth day) the latter group of macrophages had become less deeply coloured (Fig. 11) while the original oval intensely stained body persisted, until on the ninth day (Fig. 12) it alone remained, and had moved to the other side of the vessel. On the thirteenth day it was still present in this situation, although appearing somewhat paler. By the twentieth day it was no longer visible.

The exact interpretation to be given to this particular sequence of changes is not clear. It was not possible to determine if dye-complex was being passed from the macrophages into the vessel, or to the deeply stained oval body. Nor has convincing evidence on the origin of bodies of the latter type, whether derived from a macrophage by mitosis or fragmentation, or appearing in some other way, been as yet obtained.

In one animal there were appearances suggesting the staining of the endothelium of capillaries within one hour of the injection of the dye-complex. This occurred in a patchy fashion, but the staining was so faint that it was not possible to be certain that it was not due to the staining of adventitial macrophages which happend to underlie the vessels being observed, although the large size and regular outlines of the stained zones are perhaps arguments against this. In any case it must be realized that with this particular mode of injec-

tion the dye-complex would have to pass the barrier of the adventitial macrophages to reach the endothelial cells.

In another animal therefore the dye-complex was injected into a vein distal to the chamber, in the manner used in the preliminary experiments with methylene blue. Digital pressure on the vessels both at the base of the ear and in the ear margin held the solution in veins of the chamber area for approximately three minutes. The solution appeared slightly irritating, and produced an oscillatory flow in larger venules, with complete cessation in many smaller vessels. On microscopic examination of the chamber, beginning approximately five minutes after the commencement of the injection, the dye-complex was seen lying in small terminal arterioles (30μ) and in some slightly larger venules. In all cases the dye-complex was aggregated into masses, and was visible only in vessels in which there was complete cessation of blood flow. After twelve minutes one of the masses of blue staining material began to break up, and to move very slowly and jerkily down the vessel as the circulation began to be re-established. It could then be seen that several large cells, apparently monocytes or large lymphocytes, were involved in the intravascular mass. No uptake of dye-complex by vascular endothelial cells was observed even three hours later, when some of the blue-stained masses remained visible in places in vessels still showing complete interruption of blood flow.

Kruse and McMaster (1949) have noted that Evans Blue, unlinked to protein, is retained tenaciously by the macrophage. Control observations were therefore made in two animals, using a 0·75 p.c. solution of Evans Blue in 0·9 p.c. saline, injected via the access pinhole in a manner precisely analagous to that used with the Evans Blue azoprotein. With the unlinked dye vital staining of macrophages and of smaller lymphoid-like cells was observed within less than ten minutes of the injection, although the finer branches of the macrophages were not stained, and the staining was much less intense than was the case with methylene blue. It was confirmed that the dye was retained by the macrophage, and observations on subsequent days showed appearances rather like those seen with the Evans Blue azoprotein. There appeared, however, to be rather less cytoplasmic fragmentation than was the case with the azoprotein, and few small oval intensely staining bodies were observed. However, in one experiment granular blue-staining macrophages were still very evident three weeks after injection (Fig. 14) and had not entirely disappeared at six weeks.

In this animal, however, there was a small area of necrosis over the top of the pin, resulting from tissue damage at the time of withdrawal of the pin. The dye at once became tenaciously attached to the dead tissues in this area, being slowly released and engulfed by macrophages coming from the surrounding living tissue. In the other animal, where there was less trauma, few traces of the dye remained after four weeks.

In all observations with the unlinked dye the colour of the cells appeared to the eye to be a lighter blue than with the linked dye. Microspectrophotometric observations to test this point have not as yet been made, but studies with the Beckman spectrophotometer showed that there is a difference of 40mμ between the absorption peaks of the linked (570mμ) and the unlinked 610mμ) dyes.

DISCUSSION.

The most striking phenomenon observed in this work has been the rapidity and extent of the shedding of portions of the cytoplasm of the macrophage. In addition complete fragmentation of macrophages was often observed. The ingestion of the cytoplasmic fragments by neighbouring cells was a constant feature. This may represent a mechanism for the sharing of antigen among a number of macrophages. No proof has, however, yet been obtained that the dye molecule is not split off from the protein moiety of the antigen during the early stages of intracellular digestion. Rather similar appearances were in fact observed in response to the injection of Evans Blue unlinked to protein. It is known, however, that Evans Blue readily combines with albumin to form a dye-protein complex (Rawson, 1943), which may help to explain why the linked and unlinked dyes are handled by the macrophage in rather similar ways.

The presence of antigen, in the first few hours, in smaller cells resembling large lymphocytes is also of some interest. Coons and his co-workers (1950) using antibody linked to fluorescein as a specific stain were also able to locate antigen in a proportion of lymphoid cells. In the present work dye-complex was not detected in lymphocytes after the first few hours, perhaps because its concentration was then too low to be detected by this method.

Reports of the ability to shed peripheral portions of cytoplasm are not confined to the macrophage. It has also been described in lymphocytes by Downey and Weidenreich (1912) in fixed and stained sections of lymphoid organs, although Ebert, Sanders and Florey (1940) in their studies of living lymphocytes in rabbit ear chamber preparations did not observe this phenomenon. Zollinger also (1948) observed the formation of large "blisters" on the surface of cells of many types when suspended *in vitro* in saline or Ringer's solutions. These "blisters" were often cast off into the surrounding fluid. His studies did not, however, include normal lymphocytes or macrophages.

Another finding of interest in the present work was the appearance after five to six days of oval bodies in which the dye appeared to become highly concentrated. Up to the present, however, observations have not provided good evidence on their origin, although the appearances favour the possibility that they arise by fragmentation of macrophages (Figs. 8 and 13) and that they are residual nuclei which have taken up the dye. Coons, Leduc and Kaplan (1951) reported the finding of antigen within the nuclei of cells, often in apparently

higher concentration than in the cytoplasm. The cells included lymphocytes, macrophages and renal and kidney epithelial cells. They considered this was probably a real phenomenon and indicated a rapid interchange of material between nucleus and cytoplasm. The present work, however, suggests the possibility that the presence of antigen within the nucleus is most likely to occur in a degenerating or already degenerate cell. Most healthy macrophages showed no nuclear staining.

SUMMARY.

The uptake of blue antigenic azoprotein (Evans Blue linked to horse serum) has been studied in the rabbit ear chamber preparation. On injection through the access pinhole the dye-complex was very quickly fixed to the tissue. Within 2½ hours it could be seen in the cytoplasm of macrophages, and also in some cells resembling large lymphocytes. Shedding of peripheral portions of cytoplasm by macrophages was observable at 4½ hours, and this phenomenon continued in cells of this type for many days. Lymphoid cells which had ingested the antigen were not seen after the first day. The shed fragments of macrophage cytoplasm were often ingested by other neighbouring macrophages. It is suggested that this may represent a mechanism for the sharing of antigen among many macrophages. Uptake of dye-protein complex by endothelial cells was not seen even when the antigen was injected directly into the veins. In an experiment in which methylene blue solution was injected via the local venous system, adventitial macrophages became intensely stained in less than ten minutes, and shedding of cytoplasmic processes was also clearly demonstrated at that time.

Acknowledgments. I am much indebted to Mr. M. L. Welby, B.Sc., for preparing the antigens, to Miss M. Gladstone for technical assistance, and particularly to Mr. A. Humphrys, who made the chambers and much other essential equipment, and also assisted in the work in many other ways.

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THE OCCURRENCE OF SALMONELLA TYPES IN AUSTRALIA[1]. 8

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(Accepted for publication 13th September, 1951.)

In the present paper we describe another batch of Salmonella strains received for typing from all over Australia. This batch contained 750 strains of which more than half were either *S. typhi-murium* or *S. bovis-morbificans*. We had previously noted (Atkinson, Woodroofe and Macbeth, 1949) that next to *S. typhi-murium*, *S. bovis-morbificans* was the commonest type causing gastro-enteritis in Australia. The present results further emphasize the importance of this type of which 250 strains are described here, the majority of which came from a major outbreak in young children in Queensland, described by Mackerras and Mackerras (1949).

Though far below *S. typhi-murium* and *S. bovis-morbificans* in numbers, the two next commonest types from human cases of gastro-enteritis were *S. derby* and *S. adelaide*. Strains of *S. give*, *S. orion*, *S. meleagridis*, *S. kottbus*, *S. muenchen*, *S. chester*, *S. potsdam*, *S. london*, *S. amager*, *S. champaign* and *S. orientalis* were identified in Australia for the first time. For many types, strains were obtained from both human and animal material.

EXPERIMENTAL.

Methods.

The antigenic analysis of the cultures was done according to the methods given in earlier papers of this series. The identification of those types described here for the first time in Australia was confirmed by cross-absorption tests with type antiserum and the type strains.

S. paratyphi C. was distinguished from *S. choleraesuis* and *S. typhi-suis* by growth on nutrient agar, tests for H_2S production and fermentation of arabinose and trehalose.

RESULTS.

Table 1 records the types found and their number and origin.

[1] This work was partly supported by a grant from the National Health and Medical Research Council, Canberra.

TABLE 1.

Salmonellas described in this paper.

Salmonella	Total Number of Strains	Where Isolated	Source	Number of Strains	Type of illness
S. typhi-murium	303	South Australia	children (faeces)	78	gastro-enteritis
			adults (faeces)	21	gastro-enteritis
			pigs	7	normal pigs slaughtered at Abattoirs submitted dead for routine examination
			sheep	1	severe enteritis and anaemia after travelling
			guinea-pig	1	disease in laboratory stocks
		Queensland	children (faeces)	88	mainly cases of gastro-enteritis of which several died; but a few were apparently normal picked up in a survey
			adults (faeces)	4	gastro-enteritis and one normal carrier
			sheep	3	died with scours after travelling from N.S.W. to Queensland
			ducks (intestine, liver, lung)	15	epidemic disease in young birds in various flocks
			cattle (mesenteric gland, faeces, intestine, blood)	12	scouring or paratyphoid
			pigs (lung and mesenteric gland)	2	outbreaks of pneumonia killing several pigs with numerous others sick
			rats (glands, liver, spleen)	3	healthy, caught in survey of suburban rats in Brisbane
			cockroach	1	caught in hospital kitchen, normal
			nail brush	1	from gastro-enteritis ward
			sink	1	in gastro-enteritis ward
		Victoria	adults (faeces)	3	gastro-enteritis
			guinea-pigs (blood, liver, peritoneal fluid)	7	epidemic disease in laboratory stock
			mouse (liver)	1	epidemic disease in laboratory stock
			heifer (intestine, liver)	1	scouring
			ducks (bone marrow, liver, heart blood)	2	diarrhoea
		New South Wales	children (faeces)	38	mild to severe gastro-enteritis (at least two died)
			adults (faeces)	2	gastro-enteritis
			dog (faeces)	1	enteritis
			pigeon (internal organs)	1	died
		Western Australia	children (faeces)	7	gastritis
			duck (heart blood)	1	multiple lung lesions at autopsy
			sheep (lymph node, colon contents)	1	outbreak after train journey from N.S.W. to W.A.
S. bovis-morbificans	250	South Australia	children (faeces)	11	gastro-enteritis
			adults (faeces)	3	gastro-enteritis
			sheep	1	flock of 200 sheep died in 3 days after arsenical drench
		Victoria	duckling (liver)	1	diarrhoea, died
		New South Wales	children (faeces)	8	gastro-enteritis (or chronic diarrhoea)
			foal (spleen and liver)	1	died; lung abscess with *C. equi* present
		New Zealand	child (faeces)	1	gastro-enteritis
			adult (faeces)	1	fever

TABLE 1 (continued).

Salmonella	Total Number of Strains	Where Isolated	Source	Number of Strains	Type of illness
S. bovis-morbificans		Queensland	children (faeces, cerebro-spinal fluid, liver, spleen, mesenteric gland, colon, ileum, mastoid, jejunum, lung or peritoneum)	33	fatal gastro-enteritis often with complications*
			children (faeces)	2	carriers*
			children (faeces)	170	gastro-enteritis*
			ward sink	1	in hospital ward*
			scrubbing brush of napkin boiler	1	in same hospital*
			sink and nail brush	1	in hospital ward*
			cockroaches and cockroach pool	3	caught in same hospital*
			mice (liver, intestine, colon)	4	caught in same hospital*
			adults (faeces)	2	gastro-enteritis
			rat (spleen)	1	suburban rat, healthy
			cat (colon)	1	sick but no diarrhoea
			duckling (heart blood)	1	50 out of 50 died
S. adelaide	31	South Australia	children (faeces)	2	gastro-enteritis
		New South Wales	children (faeces)	7	gastro-enteritis
		Queensland	children (faeces)	19	gastro-enteritis or carriers
			adult (faeces)	1	gastro-enteritis
			rats (spleen, gut, bladder)	2	suburban rats, healthy
S. derby	24	South Australia	children (faeces)	4	gastro-enteritis
		New South Wales	children (faeces)	3	gastro-enteritis
			adult (faeces)	1	diarrhoea
		Western Australia	chicken (peritoneal fluid)	1	peritonitis
		Queensland	children (faeces)	7	gastro-enteritis
			adult (faeces)	1	carrier
			chickens (lung, liver)	5	numerous young chickens dying in four flocks
			dogs (faeces)	2	enteritis with coccidiosis, both dogs came from the same kennel
S. muenchen	22	South Australia	children (faeces)	3	gastro-enteritis
			pigs	2	normal pigs slaughtered at Abattoirs
		Western Australia	child (faeces)	1	diarrhoea and mild upper respiratory tract infection
			chicken	1	no history
		Queensland	children (faeces)	4	gastro-enteritis or pyrexia
			adults (faeces)	4	gastro-enteritis
			bulls (spleen, small intestine)	2	watery diarrhoea, died†
			chickens (lung, intestinal contents)	5	numerous young chicks dying in three flocks.

TABLE 1 (continued).

Salmonella	Total Number of Strains	Where Isolated	Source	Number of Strains	Type of illness
S. cholerae-suis var. *kunzendorf*	19	South Australia	pigs (liver, lung)	2	47 out of 136 died with joint swellings and pneumonia. Pastiwella also isolated
		Queensland	pigs (lung, spleen, liver, blood)	17	outbreaks of paratyphoid, pneumonia, all died
S. anatum	18	South Australia	adults (faeces)	7	gastro-enteritis in a mental hospital
		Queensland	adults (faeces)	5	gastro-enteritis and one carrier
			bulls (mesenteric lymph gland, small intestine, lung)	2	diarrhoea after travelling, both died
			chickens (liver, lung)	2	numerous week-old chickens dying in two different flocks
			duck (intestine)	1	17 out of 400 young ducks had died
			pig (lymph gland)	1	one out of 40 sick
S. chester	11	Queensland	children (faeces)	4	gastro-enteritis
			adults (faeces)	1	gastro-enteritis
			rat (intestine)	1	suburban rat, healthy
			pig	1	liver abscesses
			chickens	4	numerous losses in two different flocks
S. newington	10	Victoria	adult (faeces)	1	gastro-enteritis
		South Australia	elephant (faeces)	1	diarrhoea after Glauber's salts
		New South Wales	adult (faeces)	1	gastro-enteritis
		Queensland	adults (faeces)	3	healthy carrier, typhoid carrier, gastro-enteritis case
			chickens (liver)	2	numerous losses on one farm
			cow (lungs, heart)	1	died with pneumonia and pleurisy
			horse (large and small intestine)	1	died after trucking. Severe inflammation of the colon was evident at autopsy
S. newport	8	South Australia	adult (faeces)	1	gastro-enteritis arising during recent flight from London
		New South Wales	child (faeces)	1	mild gastro-enteritis
		Queensland	child (faeces, spleen)	1	gastro-enteritis, died
			chickens (lung, liver and intestine)	5	100 out of 250 died within 14 days of hatching
S. cholerae-suis	9	New Zealand	pigs	5	no history available
		South Australia	pig	1	normal pig slaughtered at Abattoirs
		Tasmania	pig	1	no history available
		Western Australia	pigs (mesenteric lymph node)	2	necrotic enteritis
S. paratyphi C	5	Queensland	rats (intestine)	2	suburban rats, healthy, caught during survey of rodents
			child (faeces)	1	gastro-enteritis
			pig (kidney)	1	pneumonia and nephritis
		Western Australia	pig (spleen)	1	20 pigs died on one property in a week with scouring and coughing

TABLE 1 (continued).

Salmonella	Total Number of Strains	Where Isolated	Source	Number of Strains	Type of illness
S. oslo	4	Queensland	adult (faeces)	1	gastro-enteritis
			chickens (lung)	3	numerous losses on three different properties
S. kottbus	5	New South Wales	children (faeces)	4	gastro-enteritis
		Queensland	adult (faeces)	1	healthy carrier
S. enteritidis	4	Victoria	mouse	1	from outbreak in mouse stock in a hospital laboratory
		Western Australia	mice (blood)	3	from epidemic in laboratory mouse colony
S. meleagridis	4	Queensland	adult (faeces)	1	gastro-enteritis
			rat (intestine)	1	healthy, caught in rodent survey
			chickens (lung and liver)	2	from one flock already recorded for *S. chester* above
S. gire	4	Queensland	adult (faeces)	1	acute gastro-enteritis
			pig	1	one out of 23 pigs died and six others were sick
			chickens (lung, liver)	2	died in outbreaks on different properties
S. orion	2	South Australia	children (faeces)	2	gastro-enteritis
S. senftenberg	2	South Australia	children (faeces)	2	gastro-enteritis
S. bareilly	2	Queensland	child (faeces)	1	healthy carrier
			chicken (liver)	1	numerous losses on one farm
S. cambridge	2	New South Wales	cat (mesenteric lymph node)	1	enteritis
		Queensland	chicken	1	numerous losses on one farm
S. paratyphi A	2	South Australia	child	1	no history
			adult	1	no history
S. oranienberg	1	South Australia	child (faeces)	1	gastro-enteritis
S. potsdam	1	South Australia	child (faeces)	1	gastro-enteritis
S. london	1	Queensland	bull (faeces)	1	mild diarrhoea
S. new-brunswick	1	New South Wales	adult (faeces)	1	gastro-enteritis
S. amager	1	Queensland	child (faeces)	1	gastro-enteritis
S. worthington	1	Western Australia	pig (mesenteric lymph gland)	1	necrotic enteritis
S. paratyphi B	1	Queensland	child (faeces)	1	diarrhoea
S. champaign	1	Queensland	chicken	1	from same flock recorded for *S. meleagridis* above
S. orientalis	1	Queensland	calf (gall bladder, liver)	1	scouring, emaciation

* The outbreak from which these strains came was described by Mackerras and Mackerras (1949).

† One of these bulls also yielded *S. typhi-murium* and *S. anatum* from mesenteric lymph glands.

Out of the total of 750 strains typed, 303 were *S. typhi-murium* and 250 were *S. bovis-morbificans*. No other types occurred so abundantly and the next most prevalent types were *S. adelaide* (31 strains), *S. derby* (24 strains) and *S. muenchen* (22 strains). From 10 to 19 strains were obtained of *S. choleraesuis* var. *kunzendorf*, *S. anatum*, *S. chester* and *S. newington*. There were from three to nine strains of *S. choleraesuis*, *S. paratyphi C*, *S. oslo*, *S. kottbus*, *S. enteritidis*, *S. meleagridis*, *S. give*, and *S. newport*. One or two strains only were obtained of *S. orion*, *S. senftenberg*, *S. bareilly*, *S. cambridge*, *S. paratyphi A*, *S. oranienberg*, *S. potsdam*, *S. london*, *S. new-brunswick*, *S. amager*, *S. worthington*, *S. paratyphi B*, *S. champaign*, *S. orientalis*. Altogether 30 different types were found.

Most of the 303 strains of *S. typhi-murium* came from human cases of gastro-enteritis but the remainder came from a variety of animals including pigs, cattle, sheep, ducks, rats, and a dog.

Of the 240 strains of *S. bovis-morbificans* over 200 came from an epidemic of gastro-enteritis in Brisbane described by Mackerras and Mackerras (1949). Most of these strains were isolated from patients, but a number of strains were also obtained from sinks, scrubbing brushes, and from cockroaches and mice in the hospital in which the outbreak occurred. This organism was also found in cases of gastro-enteritis in children in South Australia and New South Wales and two cases were confirmed for New Zealand. Rats, cats, ducks, and a foal also provided strains of *S. bovis-morbificans*.

S. adelaide was mainly found in human cases of gastro-enteritis during the Brisbane epidemic, but it was also found in Brisbane rats. *S. derby*, besides occurring in human gastro-enteritis, was found in outbreaks in chickens and dogs. *S. cholerae-suis* was found only in pigs, and *S. enteritidis* was found only in mice. *S. cambridge* occurred only in a cat and a chicken, *S. london* in a bull, *S. champaign* in a chicken, and *S. orientalis* in a calf.

The few strains each of *S. orion*, *S. senftenberg*, *S. paratyphi A*, *S. oranienberg*, *S. potsdam*, *S. new-brunswick*, *S. amager*, *S. kottbus*, and *S. paratyphi B* all came from children or adults suffering mainly from gastro-enteritis.

Most of the other types occurred in both humans and animals. *S. muenchen* was found in pigs, cattle and chickens as well as humans. *S. anatum* was responsible for an outbreak of gastro-enteritis in an institution, and also was found in pigs, cattle, chickens, and ducks. *S. chester* was only found in Queensland, where it occurred in children, adults, rats, pigs, and chickens. *S. newington* infected men, cows, horses, chickens, and an elephant. *S. newport* infected men and chickens. *S. paratyphi C* occurred in rats, pigs, and children. *S. oslo*, *S. meleagridis*, *S. give*, and *S. bareilly* were recorded only for Queensland, where they occurred in men and chickens. *S. give* was also found in a pig, and *S. meleagridis* in a healthy rat.

DISCUSSION.

The present group of cultures is larger than any previously reported by us, and contained cultures from all over Australia. It included many more types than were found in previous batches, probably on account of the inclusion of a considerable quantity of animal material.

The distribution of the types between human and animal material is given in Table 2.

TABLE 2.

Distribution of Salmonella types in human and animal material.

Salmonella	No. of strains from animals.	No. of strains from man	Total no. of strains
S. typhi-murium	60 (cattle, sheep, pigs, guinea-pigs, ducks, rats, mouse, dog, pigeon, cockroach)	241	301
S. bovis-morbificans	13 (sheep, ducks, foal, mice, rat, cat, cockroaches)	234	247
S. adelaide	2 (rats)	29	31
S. derby	8 (chickens, dogs)	16	24
S. muenchen	10 (pigs, cattle, chickens)	12	22
S. cholerae-suis (including var. *kunzendorf*)	28 (pigs)	0	28
S. anatum	6 (cattle, pig, chickens, duck)	12	18
S. chester	6 (rat, pig, chickens)	5	11
S. newington	5 (chickens, cow, horse, elephant)	5	10
S. newport	5 (chickens)	3	8
S. paratyphi C	4 (pigs, rats)	1	5
S. kottbus	0	5	5
S. oslo	3 (chickens)	1	4
S. enteritidis	4 (mice)	0	4
S. meleagridis	3 (rat, chickens)	1	4
S. give	3 (pig, chickens)	1	4
S. orion	0	3	3
S. senftenberg	0	3	3
S. bareilly	1 (chicken)	1	3
S. cambridge	2 (cat, chicken)	0	3
S. paratyphi A	0	2	2
S. oranienberg	0	1	1
S. potsdam	0	1	1
S. london	1 (bull)	0	1
S. new-brunswick	0	1	1
S. amager	0	1	1
S worthington	1 (pig)	0	1
S. paratyphi B	0	1	1
S. champaign	1 (chicken)	0	1
S. orientalis	1 (calf)	0	1

S. typhi-murium, *S. bovis-morbificans*, *S. muenchen*, *S. anatum*, *S. chester*, and *S. newington* were widely distributed among animal species as well as being responsible for human infection. Apart from human infections *S. adelaide* was

only found in rats. Chickens and ducks may harbour numerous Salmonellas, and in the present group *S. typhi-murium, S. bovis-morbificans, S. derby, S. muenchen, S. anatum, S. chester, S. newington, S. newport, S. oslo, S. meleagridis, S. give, S. bareilly, S. cambridge,* and *S. champaign* were isolated from one or both of these birds.

Thirty different types were found in the present batch of Salmonellas, but strains of *S. typhi-murium* and *S. bovis-morbificans* far outnumbered all the other types, many of which provided less than 10 strains each.

The present work emphasizes the extent of the range of Salmonellas existing in Australia, and infecting both man and a wide variety of animals.

SUMMARY.

A group of 750 Australian Salmonellas has been typed.

Thirty different types were found, of which by far the most prevalent were *S. typhi-murium* and *S. bovis-morbificans.*

Many of the types occurred in both human and animal disease.

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STUDIES OF THE FLORA OF THE MOUTH

VIII. AN EXAMINATION OF SELECTED HUMAN STRAINS OF ANAEROBIC ACTINOMYCES

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It is recognized that the anaerobic strains of the genus Actinomyces are not homogeneous but the results of attempts to describe species within this genus have not been conclusive. Breed *et al.* (1948) have divided the genus into two species: the organisms isolated from human infections and corresponding to the description by Wolff and Israel (1891), and those isolated from bovine infections and forming smoother and more regular colonies.

There are indications in the literature that the strains isolated from humans may be divided into yet other species. This paper presents the results of attempts to determine by (I) morphological, (II) physiological and (III) serological means whether the group of human strains is homogeneous and whether it bears any relationship to strains isolated from bovine infections.

EXPERIMENTAL PROCEDURES.

Isolation and Selection of the Organisms.

Materials and methods.

Materials. (a) The organisms to be studied were isolated from pus from actinomycotic lesions occurring around the oral cavity of persons attending the United Dental Hospital of Sydney. The pus was obtained following upon the extra-oral incision of such lesions.

(b) One organism was isolated from a lesion in a patient suffering from thoracic actinomycosis.

(c) A strain of Actinomyces isolated from a bovine infection was supplied by Professor H. R. Carne of the Faculty of Veterinary Science, University of Sydney.

Methods. The samples of pus were examined for the presence of "sulphur" granules. A selected granule from each sample was washed in a small quantity of sterile nutrient broth, spread on plates of 2·5 p.c. nutrient agar containing 8 ml. blood per 100 ml. medium and one plate incubated anaerobically and another aerobically at 37° C. for 7 days. Separate colonies were picked and transferred to blood agar, then subcultured in 10 p.c. serum broth. After tests for purity had been carried out, the organism was transferred to Robertson's cooked meat medium and stored in a refrigerator. In this way thirteen strains of Actinomyces were isolated from suppurative cervico-facial lesions in man and one from a human thoracic lesion.

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The organisms were found only when the material was cultivated under anaerobic conditions. With the technique described little difficulty was experienced in obtaining pure cultures. As already mentioned, a bovine strain was obtained for comparative studies.

All tests described in the following sections were carried out three times.

CULTURAL AND MORPHOLOGICAL CHARACTERISTICS.

Materials and methods.

Cultural characteristics. (a) Growth in a liquid medium was studied after 4 and 7 days' anaerobic incubation at 37° C. in 10 p.c. serum broth.

(b) Growth on a solid medium and colony formation were studied after 4 and 7 days' anaerobic incubation at 37° C. on 2·5 p.c. nutrient agar containing 10 p.c. horse serum.

Morphology. (a) Films stained by Gram's method were prepared from 7-day cultures in serum broth and on serum agar, and studied.

(b) Surface agar slide cultures were studied after 1, 2, 3 and 4 days' incubation. The organisms were grown on serum agar upon slides in sealed petri dishes, which contained also a pyrogallol-alkali-keiselguhr mixture. In addition the organisms were incubated for as long as a month and periodically examined during that time.

(c) "Buried" slide cultures were prepared in the following manner: 1 ml. liquid 10 p.c. serum agar was seeded with two drops of a 4-day broth culture of the organism to be examined. This seeded agar, whilst still molten, was run onto a sterile glass slide, the film being kept as thin as possible. After the agar had solidified, the slide was immersed in a tube of broth containing 10 p.c. serum and the preparation was incubated anaerobically for 24 hours. It was removed from the broth and dried very slowly upon a warm oven. The preparation was then stained by the following method:

Apply aniline gentian violet for 3 minutes; apply Gram's iodine for 2 minutes; decolourize very slowly with aniline oil; wash in xylol and dry. Other, more rapidly-acting decolourizing agents tended to remove the stain from the organism more quickly than from the agar.

(d) Acid-fastness was tested by the Ziehl-Neelsen method, using as a decolourizing agent 0·5 p.c. sulphuric acid applied for 30 to 60 seconds.

Cultural characteristics.

Growth in a liquid medium. In all cases the growth of the organism occurred at the bottom of the tube. The type of growth varied from coarse, lumpy granules, through feathery and finely granular deposits, to very smooth and even mucoid deposits. Where a smooth deposit occurred, there was at times some diffusion of growth throughout the medium. Such diffusion was never particularly heavy, except in the case of the bovine strain. It was noted that the age of the parent culture and the amount of serum in the medium caused slight variations in the type of the growth of the subculture.

Growth on a solid medium. Eleven of the strains grew initially as colonies similar to those of classical strains of Actinomyces. They were smooth, opaque, white, hemispherical masses with a central umbilication and they were often irregular in shape. Usually they were cartilagenous in consistency and somewhat sunken into the medium. This description is identical with that given by Wolff and Israel (1891).

After these strains had been periodically subcultured for some months, seven still grew in this characteristic, rough form. However, the remaining four showed varying tendencies to become smoother and non-adherent and are described as intermediate strains throughout this paper.

Three strains grew initially as smooth colonies. The colonies of one of these closely resembled the smooth colonies produced by the four intermediate strains. The other two strains developed colonies up to 1·5 mm. in diameter and grew more rapidly, developing their maximum size within 48 hours.

The colonies produced by the bovine strain were smooth and almost identical with those of the smooth strains of human origin.

Morphology.

Microscopic appearance. Most of the strains showed irregularly staining, Gram-positive filaments of varying length. The rough strains produced longer filaments than the smooth strains, which often assumed a diphtheroid form. Branching was difficult to detect, so the use of such stained films was not considered reliable for the investigation of this characteristic.

Surface agar slide cultures. All strains showed an undivided mycelium which later fragmented. Three patterns of growth were observed.

The strains which constantly formed rough colonies without any tendency to produce smoother colonies grew as long, branching filaments disposed in a radial fashion. Branching was monopodial. As the mycelial structure developed, certain upgrowths were seen which may have corresponded to the aerial hyphae described by Erikson (1940) but no firm conclusions could be reached on this point.

The strains which showed rough to smooth variation had shorter filaments. They seemed to fragment more rapidly and no aerial structures were noted.

The smooth strains closely resembled the intermediate strains but seemed to fragment even more readily and often had the appearance of diphtheroid rods. Such was the conformation that it was often difficult to decide whether true branching existed or not. No aerial structures were seen.

The bovine strain was in no way different from the smooth strains already described.

All strains could be included in Group IIB of Orskov's (1923) classification.

"Buried" slide cultures. This method proved very effective for examining the arrangement of the organisms. The organism conformed with the growth patterns seen in the agar slide preparations but branching was far more readily observed. This applied in particular to the intermediate and smooth strains which appeared as short filaments with numerous diphtheroid forms. However, short branches could be seen always.

Acid-fastness. None of the strains was acid-fast.

Physiological Characteristics.

Materials and methods.

Oxygen requirements. The organisms were seeded onto serum agar plates and incubated at 37° C. under both anaerobic and aerobic conditions. The plates were examined for growth at the end of 4, 7 and 14 days.

In addition serum-agar shake-cultures were prepared and the depth at which the organisms grew optimally within the medium was noted.

Fermentation reactions. Tests were carried out to determine the ability of the organisms to ferment glucose, sucrose, maltose, lactose, dextrin, starch, inositol, glycerol, mannitol, dulcitol, inulin, glycogen and salicin. The medium used was similar to that described previously by Sullivan and Goldsworthy (1940). A very large inoculum was used because of the granular nature of the suspensions and the slow rate of growth of the organisms. The cultures were incubated at 37° C. under anaerobic conditions and readings were made on the seventh and fourteenth days. Control tubes in which there was no added substrate were incubated at the same time.

Growth on potassium tellurite medium and Loeffler's medium. The ability of the organisms to grow upon a medium containing nutrient agar, glycerolate of blood and potassium tellurite and upon Loeffler's inspissated serum medium was tested under both aerobic and anaerobic conditions. When growth occurred, films were prepared and examined after staining by Gram's and Neisser's methods.

Strains of *C. diphtheriae* and *C. hofmannii* were used as control organisms.

Oxygen requirements.

All strains preferred anaerobic conditions for growth but, after frequent subculturing, most strains seemed to develop some degree of tolerance to aerobic conditions. This was more noticeable in the smooth and intermediate strains than in the rough strains, but occasionally some of the rough strains grew scantily under aerobic conditions. After maintenance in the laboratory for some time two of the smooth strains grew as well under aerobic conditions as they did under anaerobic conditions and the bovine strain grew freely under both anaerobic and aerobic conditions.

The growth of the organisms in serum-agar shake-cultures generally confirmed the results already described. Although discrete colonies occurred at different levels in all cultures, the zone of maximum growth in the rough and intermediate types was situated between one and two centimetres below the surface. The two smooth strains referred to above showed a maximum growth centre only slightly below the surface of the medium.

It was interesting to note that the zone of maximum growth of the bovine strain was situated deeply within the medium.

Fermentation reactions. (See Table 1).

The fermentation reactions of the individual strains varied considerably. All strains except the bovine strain fermented glucose, sucrose, lactose, maltose, dextrin and inositol. Salicin was fermented by all strains with the exception of two of the intermediate strains and one smooth strain. Starch, inulin and mannitol were fermented in varying degrees by some strains. Glycerol was fermented by one smooth strain only and dulcitol was not fermented by any.

TABLE 1.

Fermentation reactions of fifteen selected strains of anaerobic Actinomyces.

Organisms grouped according to morphology	Rough							Intermediate				Smooth			Bovine	Control
	2	4	5	8	11	13	16	7	10	14	15	1	6	12	*A. bovis*	No organisms
Glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−
Sucrose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−
Maltose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−
Lactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−
Dextrin	+	+	+	+	+		+	+	+	+	+	+	+	+	−	−
Starch	−	−	−	−	−		−	+	+	−	−	+	+	+	−	−
Glycerol	−	−	−	−	−		−	−	−	−	−	+	−	−	−	−
Mannitol	−	+	+	−	−		−	−	+	−	−	−	−	+	−	−
Inositol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−
Dulcitol	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
Inulin	−	−	−	−	−	−	−	−	−	−	−	+	−	+	−	−
Glycogen	+	+	+	+	+	+	+	+	+	+	+	+	+	+	−	−
Salicin	+	+	+	+	+	+	+	+	+	−	−	+	−	+	−	−
Control (no sugar)	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

All readings represent mean value obtained from three testings of the fermentation reactions of the organisms.

The bovine strain failed to ferment the majority of the substrates tested. Maltose and lactose were the only sugars broken down.

Growth on potassium tellurite medium and Loeffler's medium.

None of the strains grew on a medium containing potassium tellurite. The strains of *C. diphtheriae* and *C. hofmannii*, which were used as controls, grew well.

Most of the organisms failed to grow on Loeffler's medium, although some strains occasionally grew slightly. The corynebacteria grew well.

Serological Characteristics.

Materials and methods.

Preparation of antigen suspensions. Five strains were chosen: a typical rough strain, a typical intermediate strain, a typical smooth strain, the strain isolated from a thoracic lesion and the bovine strain. Each strain was grown in a flask containing 250 ml. nutrient broth under anaerobic conditions for 7 days at 37° C. The resultant growth was collected and suspended in a small quantity of 0·5 p.c. carbol-saline. This suspension was frozen and then ground with a sterile glass rod as it thawed.

Standardized suspensions of the organisms in 0·5 p.c. carbol-saline were then prepared. The density of the suspensions was comparable with that of a suspension of *B. coli* containing 2,000 million organisms per ml. Sufficient quantities were made for the whole experiment and they were then stored in sterile containers in the refrigerator.

Preparation of antisera. Fifteen rabbits were bled from the ear in order to obtain samples of normal serum to be tested for already existing specific antibodies. The rabbits were then divided into five groups of three and the rabbits in each group received injections of the same suspension. They were given intravenous injections (ear vein) of 1 ml. of the suspension every fourth day, six injections in all. One week after the last injection they were bled by cardiac puncture. The rabbits were then given a "booster" dose of 1 ml. of the suspension and, after ten days' rest, were bled again by cardiac puncture. One rabbit died before the series of injections was completed and thus only two samples of antisera against the bovine strain were obtained.

Preparation of bacterial suspensions. The preparation of suitable suspensions for the agglutination reactions proved extremely difficult. Various methods were tried, including the addition of a detergent to the culture medium (Tween 80) and tryptic digestion of the organisms. None of these techniques proved satisfactory. Our suspensions were therefore prepared in the following manner: the organisms were grown in 500 ml. nutrient broth containing 0·5 p.c. sodium thioglycollate at 37° C. for 7 days. The growth was concentrated and, after freezing and grinding, was suspended in 0·4 p.c. saline. The concentration of the suspensions was adjusted so that all were comparable.

Preparation of specific carbohydrate fractions. The strains were grown in 500 ml. nutrient broth containing 0·5 p.c. sodium thioglycollate for 7 days at 37° C. The cultures were centrifuged and the supernatant fluid removed. Specific carbohydrate fractions were then prepared from the organisms by the formamide extraction technique described by Fuller (1938).

Serological properties of the organisms. All strains were tested by both precipitation and agglutination techniques against the fifteen samples of antisera which were obtained after the first course of injections. It was impossible to carry out confirmatory tests, firstly, because of insufficient quantities of the antiserum obtained after the first course of injections and, secondly, because of the inadequate concentration of agglutinins in the antisera obtained after the "booster" doses had been given.

TABLE 2.

The degree of agglutination of selected strains of anaerobic Actinomyces by antisera collected after primary course of injections.

Organisms grouped according to morphology		Rough							Intermediate				Smooth			Bovine
		2	4	5	8	11	13	16	7	10	14	15	1	6	12	*A. bovis*
Rough	Antiserum A (Strain 4)	1/2,560	1/1,280	1/320	1/160	1/1,280	—	1/1,280	—	1/160	*	—	*	—	*	—
	Antiserum B (Strain 4)	1/2,560	1/2,560	1/1,280	1/160	1/2,560	—	1/320	—	1/320	*	—	—	1/80	*	1/320
	Antiserum C (Strain 4)	1/2,560	1/2,560	1/1,280	1/160	1/2,560	—	1/1,280	—	1/160	—	—	—	—	*	—
	Antiserum D (Strain 13)	—	1/320	—	—	—	1/2,560	—	—	—	—	—	*	—	*	—
	Antiserum E (Strain 13)	—	1/80	—	—	—	1/2,560	—	—	—	—	—	*	—	—	—
	Antiserum F (Strain 13)	—	1/80	—	—	—	—	—	—	—	—	—	—	—	—	—
Pleomorphic	Antiserum G (Strain 15)	—	—	1/40	—	—	—	—	1/2,560	—	—	1/2,560	—	1/1,280	—	—
	Antiserum H (Strain 15)	—	—	1/160	—	—	—	—	1/2,560	—	—	1/2,560	—	1/1,280	—	—
	Antiserum I (Strain 15)	—	—	1/160	—	1/320	*	—	1/2,560	—	—	1/2,560	—	1/2,560	*	—
Smooth	Antiserum J (Strain 1)	—	—	—	—	—	—	—	*	—	*	1/320	*	1/320	*	—
	Antiserum K (Strain 1)	—	*	—	—	—	*	—	1/320	—	*	1/320	*	1/2,560	*	1/40
	Antiserum L (Strain 1)	—	—	—	—	*	—	—	1/320	—	*	1/320	—	1/320	*	1/160
Bovine	Antiserum M (*A. bovis*)	—	1/160	—	—	1/640	—	—	—	—	—	1/320	—	—	—	—
	Antiserum N (*A. bovis*)	—	—	—	—	—	—	—	—	—	—	1/160	—	—	—	—

* Spontaneous agglutination of the bacterial suspensions in these tubes prevented the recording of a reading.

(a) Agglutination reactions. Saline dilutions were made of the antisera (1: 40—1:2,560). To 0·5 ml. of each dilution was added 0·5 ml. of the suspension of the organism to be tested. The tubes were then placed in a waterbath maintained at 56° C. and readings were made at the end of 15 minutes, 1 hour, 2 hours and 4 hours.

(b) Precipitation reactions. Small quantities of the antisera were placed at the bottom of precipitation tubes (2–2·5 mm. bore) and the formamide extract of the organism carefully superimposed. At the end of 15 minutes, 30 minutes and 1 hour, the tubes were examined for the appearance of precipitate at the interface of the serum and extract.

Agglutination reactions. (See Table 2.)

An examination of the normal sera by both slide and tube agglutination failed, within the limits discussed below, to reveal the presence of any specific antibodies.

The interpretation of the agglutination reactions was difficult. The granular growth of a number of strains prevented the preparation of completely homogeneous suspensions of the organisms despite numerous attempts. In addition, although the organisms were diluted in 0·2 p.c. saline, spontaneous agglutination occasionally occurred in the control suspensions. The antisera collected after the preliminary course of injections gave quite adequate antibody titres with the homologous strains, with the exception of the antisera prepared against *A. bovis*. A satisfactory reading could not be made of the reaction of the selected smooth strain and its homologous antiserum, because of spontaneous agglutination. However, an adequate reading was obtained with one of the other smooth strains.

As a general observation, agglutination reactions divided the strains roughly into groups corresponding to their morphological groups but considerable cross-reaction did occur. It was not possible to determine whether any serological relationship existed between the human and bovine strains, because of the absence of any effective agglutinins in the antisera for the bovine strain.

Precipitation reactions. (See Table 3.)

It will be seen from Table 3 that quite specific reactions were obtained with the first antisera. With the exception of one intermediate strain,[2] the rough, intermediate and smooth organisms constitute three distinct groups. Furthermore, the specfic carbohydrate fraction prepared from the bovine strain gave no cross-reaction with the antisera prepared against the human strains.

The fraction from the bovine strain gave good reactions with the antisera prepared against the bovine strain. The fractions from three of the human strains gave a cross-reaction with one of the antisera for the bovine strain.

[2] This strain (strain 10) was originally classified as an intermediate type. Its variation from the rough strains was not great but, as some variation did occur, it was felt that it should not be classified as a rough strain. However, after an inspection of the serological results, it would appear that this original decision may have been at fault.

TABLE 3.

Precipitation of specific carbohydrate fractions from anaerobic Actinomyces by antisera collected after primary course of injections.

Organisms grouped according to morphology		Rough							Intermediate				Smooth			Bovine
		2	4	5	8	11	13	16	7	10	14	15	1	6	12	*A. bovis*
Rough	Antiserum A (Strain 4)	—	+	—	+	+	—	+	—	—	+	—	—	—	—	—
	Antiserum B (Strain 4)	+	+	+	+	+	+	+	+	+	—	—	—	+	—	—
	Antiserum C (Strain 4)	+	+	—	—	+	+	+	—	—	—	—	—	—	—	—
	Antiserum D (Strain 13)	+	+	—	—	+	+	+	—	+	—	—	—	—	—	—
	Antiserum E (Strain 13)	+	+	+	+	+	+	+	—	—	—	—	—	—	—	—
	Antiserum F (Strain 13)	+	+	+	+	+	+	+	—	+	—	—	—	—	—	—
Pleomorphic	Antiserum G (Strain 15)	—	—	—	—	—	—	—	+	—	+	+	—	—	—	—
	Antiserum H (Strain 15)						no		reactions							
	Antiserum I (Strain 15)	—	—	—	—	—	—	—	+	—	+	+	—	—	—	—
	Antiserum J (Strain 1)						no		reactions							
Smooth	Antiserum K (Strain 1)	—	—	—	—	—	—	—	—	—	—	—	+	+	+	—
	Antiserum L (Strain 1)	—	—	—	—	—	—	—	—	—	—	—	+	+	+	—
Bovine	Antiserum M (*A. bovis*)	—	—	+	+	—	—	—	—	+	—	—	—	—	—	+
	Antiserum N (*A bovis*)	—	—	—	—	—	—	—	—	—	—	—	—	—	—	+

DISCUSSION.

The classification of anaerobic actinomyces.

In 1891 Wolff and Israel published results of a study of the pathogenic action of the microaerophilic Actinomyces and accepted it as the specific causative organism in human and bovine actinomycosis. Since that time a number of attempts have been made to describe species within the genus Actinomyces. Colebrook (1920) examined a number of strains of human origin and described two groups, the first of which was composed of rough strains and corresponded to the description given by Wolff and Israel. The second group was composed of organisms which produced smooth growth-forms.

Holm (1930), after examining nine human strains, divided them into two groups based on the fermentation reactions and serological characteristics of the organisms. He did not describe their morphology. Lentze (1938) described cultural, serological and biochemical characteristics as a basis for grouping his strains of human origin. On a morphological basis he distinguished two types, rough and smooth. He described many difficulties encountered in his serological techniques and found that the R group could be divided into three types, whilst the S group was apparently more homogeneous. There was no obvious serological relationship between the two groups. Erikson (1940) made a detailed investigation of fifteen strains of human origin and five strains isolated from bovine lesions. The human strains, whose cultural characteristics were similar to those described by Wolff and Israel, were quite distinct both morphologically, culturally and physiologically from the bovine strains. She also stated that, while the two groups were serologically homogeneous, they bore no relationship to each other. Erikson employed agglutination reactions as a basis for these observations. She then described two species within the genus Actinomyces, namely *A. israeli*, which included all strains isolated from human lesions, and *A. bovis*, which included all strains isolated from bovine lesions. Thompson (1950), in a short report, supported this classification, basing his observations upon the morphological and cultural characteristics of some human and bovine strains.

Morphological and cultural characteristics.

It was noted early in our investigations that a few of the strains which we had isolated bore some morpohogical resemblance to corynebacteria. We therefore tested the ability of the organisms to grow upon media containing potassium tellurite and upon Loeffler's medium. In view of the fact that all strains gave practically no growth and that in addition by the use of slide cultures, it was always possible to detect true branching, we felt confident in assuming that all strains were true Actinomyces. This was supported by the fact that upon primary isolation all strains grew only under anaerobic conditions.

In our study of fourteen strains isolated from human lesions it was possible to recognize three morphological types. The first type conformed to the description usually given of a typical human strain of Actinomyces. It also seemed to be identical with the rough strains described by Erikson. Although we followed her methods, we could not convince ourselves of the presence of

any aerial hyphae such as she states were sometimes present in cultures of human strains but absent from cultures of bovine strains.

The second type initially was similar to the rough strains but after subculture showed a tendency to variation and produced smooth growth-forms, both in liquid and on solid media. Rosebury (1944) has shown that this could be induced experimentally by the rapid and frequent subculture of rough, human strains.

The third type gave smooth growth-forms similar to those described by Colebrook (1920) and Lentze (1938).

The bovine strain closely resembled the third type of human strain not only in its microscopic appearance but also in its cultural characteristics.

Physiological characteristics.

The degree of variation of the oxygen requirements of the Actinomyces has already been examined by Erikson (1940) and Rosebury (1944) and both have stressed the fact that the organisms are not strictly anaerobic and may show different degrees of tolerance to an atmosphere containing free oxygen. These observations were borne out by our own investigations. It was noted that even some of the rough strains, after being maintained in the laboratory for a period, would give a scanty growth upon serum agar plates when incubated under aerobic conditions. The rough strains, however, did not exhibit as much tolerance to aerobic conditions as the intermediate and smooth types. When serum-agar shake-cultures were prepared the rough types showed preference for deeper zones in the tube than did either of the other types.

The bovine strain possessed a tolerance to aerobic conditions similar to that of the human smooth type but when grown in serum-agar shake-cultures its optimum growth occurred deep within the medium.

The fermentation reactions of the individual strains varied considerably. The ability of the strains to ferment different sugars did not appear to bear any relationship to their morphological or cultural characteristics. It will be noted, however, that all strains fermented a wide variety of carbohydrates which from our observations seems to be a distinguishing feature between the human and bovine strains.

Serological characteristics.

The technical difficulties which we found associated with the serological study of Actinomyces proved to be considerable. Firstly, the granular growth of the organisms made it extremely difficult to obtain uniformly smooth suspensions for the agglutination reactions. Secondly, although the antisera obtained after an intensive series of injections possessed a moderate agglutinating titre with the homologous strain, those obtained after a "booster" dose had been given proved to be both nonspecific and of a low agglutinating titre. This

is at variance with the result given by Erikson (1940), who found a high agglutinating titre irrespective of whether a single course of injections or a course of injections and an additional "booster" dose had been given.

We are not aware of any investigations in which the specific carbohydrate fractions of the strains have been employed as a means of differentiation. Provided that an antiserum which has been obtained soon after an intensive course of injections is used, this technique has proved efficient and results obtained are easily interpreted.

The agglutination reactions (set out in Table 2), as far as the results could be interpreted, indicate that the rough and smooth strains are serologically different. However, cross-agglutinations frequently occurred within the group of the rough type and also within the group of the smooth type. Because of the absence of any agglutinating property for its homologous strain we were unable to study the serological relationship existing between the bovine and the human strains.

An examination of the precipitation reactions in Table 3 indicates again that the human strains fall into three serological groups, composed of the rough, the intermediate and smooth strains. In these experiments a clear-cut reaction was obtained with the bovine strain and its homologous antiserum. A few cross-reactions were obtained with a number of human rough strains and the antiserum for the bovine strain but generally it seemed that the bovine strain was serologically distinct from the human strains.

Conclusions.

It would appear from our investigations that morphologically, culturally and serologically three types of human strains of pathogenic, anaerobic Actinomyces can be recognized. These can be distinguished morphologically as rough, intermediate and smooth types.

Although the human smooth strains culturally and morphologically resemble a strain isolated from a bovine infection, none of the human strains could be identified serologically with the bovine strain.

The physiological properties of the human strains are too variable to permit their use in any classification. It was noted, however, that the human strains were more active physiologically than the bovine strain examined.

A strain isolated from a thoracic lesion in man was identical with the rough strain isolated from oral lesions.

We have examined only fourteen strains and realize that the number of organisms included in this study limits the value of any conclusions that may be drawn. It would seem, however, that our work and that of Colebrook (1920) and Lentze (1940) indicate that the strains of Actinomyces isolated from human lesions are not an homogeneous group. Whilst material from the majority of lesions yields rough and intermediate forms, occasionally smooth

forms are isolated. These smooth forms bear no serological relationship to the morphologically similar bovine strain so that they cannot be fitted into the classification of the Actinomyces adopted by Breed *et al.* (1948).

It is of interest to record that in the routine examination of these Actinomyces from human lesions all proved to be penicillin-sensitive.

SUMMARY.

The morphological, physiological and serological properties of fourteen strains of anaerobic Actinomyces isolated from human lesions have been studied.

The properties of these organisms have been compared with those of an anaerobic strain of Actinomyces isolated from a bovine lesion.

Whilst our results are not conclusive, we have presented evidence based upon morphological, cultural and serological characteristics for the existence of three types, namely, rough, intermediate and smooth.

Although the human smooth strains resemble the bovine strain morphologically and culturally, the human strains appear to have little serological relationship with the bovine strain.

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THE VENTILATORY RESPONSE TO LOW CONCENTRATIONS OF CARBON DIOXIDE IN NORMAL YOUNG MEN

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The presence of low concentrations (up to about ten per cent.) of carbon dioxide in the inspired air has the effect of increasing ventilatory minute volume.

Breathing amplitude rather than rate is the component of respiration which is increased by CO_2 according to Scott (1920), Barcroft and Margaria (1931), and Dripps and Comroe (1947), while Peabody (1915) holds that whether increases in rate or depth are more pronounced depends on the particular subject.

The effects of CO_2 (up to six per cent. in the inspired air) may be augmented, so some investigators claim, by higher oxygen percentages than are normally found in the atmosphere. Thus, Shock and Soley (1940) report that the effect on ventilation is greater when CO_2 is diluted with pure oxygen than when it is diluted with air, and Davies, Brow and Binger (1925) found a very slightly greater ventilatory response to CO_2 mixed with high oxygen percentages, on a single subject. Although they do not state it, Dripps and Comroe (1947) also probably believe that the highest ventilatory response to CO_2 is elicited with high oxygen percentages, for in their work with 7·6 and 10·4 p.c. CO_2, they used oxygen. However, Heller, Killiches and Drinker (1929) diluted CO_2 with air, claiming that oxygen and CO_2 had no greater effect on ventilation. Lindhard (1911), with three subjects, reported that for a given CO_2 percentage, there is an inverse relationship between the amount with which it is mixed, and the ventilatory minute volume produced.

Haldane and Priestley (1905) obtained data by calculating the ventilatory increment in two subjects on the basis of the measured increase in their alveolar CO_2 concentrations, when inspiring CO_2. However, these data were obtained only as a side issue to their main experiment, which was an endeavour to find "the alveolar CO_2 with varying percentages of CO_2 in the air inspired", and should not perhaps be judged too critically.

[1] This work was carried out at the suggestion of Professor F. S. Cotton, as part of a programme of research financed by the Joint Coal Board, appointed by the Commonwealth of Australia and the New South Wales Government.

Campbell, Douglas, Haldane and Hobson (1913) based similar conclusions to these of Haldane and Priestley upon similarly obtained data and calculated results.

Methods.

(a) The apparatus used in each determination in the present investigation consisted of two Douglas bags, a gas meter (for measuring volumes), a standard Douglas bag mouthpiece with one-way valve, a nose clip, two lengths of wide-bore rubber tubing each two feet eight inches in length, and an air compressor and a cylinder of carbon dioxide.

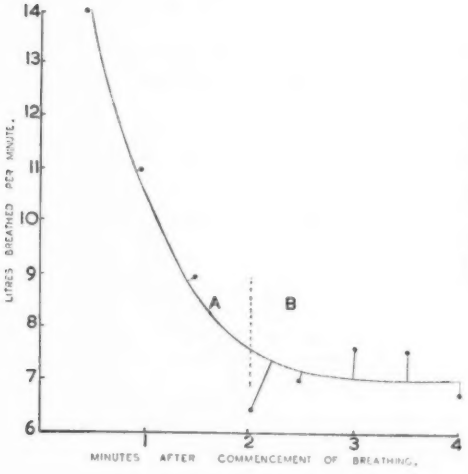

Fig. 1.

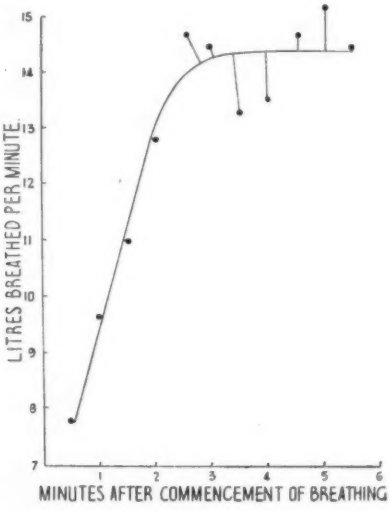

Fig. 2.

The apparatus was set up so that the subject inspired the air or gas mixture from the Douglas bags, and expired through the gas meter. In this way it was possible to predetermine the composition of the inspired air and to measure the volume of air expired.

(b) The subjects were rested for some ten to fifteen minutes before beginning each experiment; sometimes the period was slightly longer than this. No attempt was made to secure a basal condition, but attempts were made to bring each subject to the same comfortably resting state.

The bags were filled with fresh atmospheric air from the compressor, and the subjects, with mouthpiece in place and nose clip on, breathed from the bags for some minutes, the exact time varying with the individual, and depending upon the time taken to relax and reach a constant ventilation rate.

Most subjects showed the type of response shown in Fig. 1, which is drawn from the data of one of the subjects.

Section A of the breathing period, when a subject's ventilation was decreasing, was discarded and Section B, when it had reached a relatively constant rate, was taken as the subject's

resting ventilation rate. The ventilatory volume was read to the nearest tenth of a litre each half minute, so that it was easy to determine when a relatively constant ventilatory rate had been achieved. This level generally consisted of about six or seven consecutive half-minute readings. Thus it can be seen that because this method of waiting for a constant ventilatory base line was used, it was not necessary to take the resting ventilation for a very long period, and one could be reasonably certain when the true resting values had been reached. Relative constancy was considered to have been achieved when there was not more than 10 p.c. variation from the mean over a set of readings, but, of course, some subjects were much more variable than others, necessitating a slightly elastic standard.

During this part of the experiment, as a control, the subjects breathed air from the bags (as in the subsequent breathing of CO_2) rather than from the atmosphere, so that there might be complete consistency in technique.

(c) Preparation of CO_2. Three mixtures of air and CO_2 were made for each subject, such that the approximate respective percentages were 2, 4 and 6. This was done by measuring approximately 2, 4·8 and 7·2 litres of CO_2 and making up to, respectively 100, 120 and 120 litres, with air, to make mixtures of 2, 4 and 6 p.c. The contents of the bags were thoroughly mixed to ensure homogeneity. In each case an analysis was made to determine the exact percentage.

The subjects, connected as with air alone to the Douglas bags, breathed the mixtures of CO_2 and air. To eliminate any chances of psychological "preparedness" for the higher concentrations, the subjects were told they might receive the mixtures in any order. However, they always began with the 2 p.c. mixture and finished with the 6 p.c. This procedure was adhered to throughout, so that the effect of a higher percentage of CO_2 could have no effect upon the subsequent breathing of a lower percentage. The time interval between the breathing of the three percentages was always several minutes.

EXPERIMENTAL.

Effects of CO_2.

Ventilation increased initially when subjects breathed CO_2–air mixtures.

These increases are a complex, caused by three, possibly more, interacting factors.

1. The time for the air in the dead space, which fills the tube between the tap of the Douglas bags and the mouthpiece, to be used up, delays the CO_2 reaching the subject.

2. The CO_2 must be transported to the respiratory centre by the blood stream after it has entered the lungs.

3. It takes some time for all the blood to become saturated with CO_2 at the particular tension of CO_2 in the alveolar air and affect the respiratory centre to the fullest extent.

So, typically, ventilation rate increased over the resting level fairly sharply at first, then more slowly, finally reaching a plateau. This is shown in Fig. 2 which is drawn from the data of one of the subjects.

For the purposes of this investigation a plateau was defined as a set of consecutive ventilation rates (recorded at half-minute intervals) whose maximum deviation from their mean was $\pm$ 10 p.c.. At least this was the ideal aimed at. Also, except in three cases, (with the approximately 6 p.c. CO_2 mixtures), the minimum plateau length was four consecutive half-minute volumes (after Dripps and Comroe). These three were of three consecutive half-minute volumes.

The quality of the plateaux was graded as a, b and c, where those of grade a fulfilled our initial criteria as to constancy, grade b just failed, and grade c failed somewhat more, but was not more than $\pm$ 15 p.c.

These gradings will be referred to again shortly.

The results for the effects of CO_2 on ventilation rate are recorded here in Table 1.

TABLE 1.

Showing the effect of inspired CO_2 on ventilation rate.

Subject No.	Breathing air alone	Approx 2 p.c. A	Approx 2 p.c. V	Approx. 4 p.c. A	Approx. 4 p.c. V	Approx. 6 p.c. A	Approx. 6 p.c. V
1	7·2	2·31	7·7	4·03	12·7	6·10	19·8
			+ 7		+ 76		+ 175
2	10·5	1·62	11·9	3·89	23·5	5·06	29·5
			+ 13		+ 124		+ 181
3	7·2	2·10	10·1	3·95	13·2	5·87	23·7
			+ 40		+ 83		+ 229
4	7·2	4·26	23·3	5·14	28·9	7·36	42·4
			+ 224		+ 301		+ 489
5	5·9	2·17	7·7	3·88	11·0	5·26	16·8
			+ 31		+ 86		+ 185
6	9·0	1·68	13·5	3·89	17·0	5·19	23·0
			+ 50		+ 89		+ 156
7	7·7	2·23	9·8	3·96	19·1	6·48	38·4
			+ 27		+ 148		+ 399
8	8·7	2·18	9·8	4·09	18·7	6·15	36·3
			+ 13		+ 115		+ 317
9	12·6	1·54	13·7	3·90	14·4	5·74	18·5
			+ 9		+ 14		+ 47
10	7·1	1·94	9·5	4·16	14·3	5·86	24·1
			+ 34		+ 101		+ 239
11	5·4	1·84	8·1	3·97	13·9	4·95	17·0
			+ 50		+ 157		+ 215
12	9·3	2·10	12·6	3·59	17·9	5·64	26·3
			+ 35		+ 92		+ 183
13	5·1	1·96	8·6	3·87	12·0	5·83	32·5
			+ 69		+ 135		+ 537
14	8·1	2·30	11·9	4·02	19·4	5·51	32·1
			+ 47		+ 140		+ 296
15	6·9	2·03	10·0	4·04	16·2	6·17	33·2
			+ 45		+ 135		+ 381
16	9·2	1·66	12·4	4·18	14·9	5·50	21·5
			+ 35		+ 62		+ 134
17	7·3	2·29	10·4	4·23	12·5	7·36	42·4
			+ 42		+ 71		+ 190
18	8·1	2·07	11·2	3·80	14·6	4·90	20·1
			+ 38		+ 80		+ 148
19	7·2	2·00	9·4	4·14	17·2	5·81	23·0
			+ 31		+ 139		+ 219
20	7·1	2·59	11·2	3·71	13·2	5·70	16·1
			+ 58		+ 86		+ 127
21	7·5	2·21	12·8	3·73	20·3	5·99	31·0
			+ 71		+ 171		+ 313
22	6·3	1·84	9·4	3·99	16·6	5·29	23·3
			+ 49		+ 163		+ 270
23	8·5	1·70	11·8	3·52	15·3	6·03	28·7
			+ 39		+ 80		+ 238

In Table 1, A corresponds to *actual* percentage of CO_2 breathed, as revealed by gas analysis, and V corresponds to ventilation in litres per minute. Underneath these two figures, for each subject, is given the percentage increase in ventilation over the resting level.

According to the criteria already laid down, the various plateaux were classified and their gradings are collected in Table 2.

TABLE 2.

Type of plateau attained.

Subject	Breathing air alone	Approx. 2 p.c.	Approx. 4 p.c.	Approx. 6 p.c.
1	a	a	b	c
2	c	b	a	c
3	a	a	a	a
4	a	a	a	a
5	a	b	a	a
6	a	a	b	a
7	c	a	a	a
8	a	a	a	b
9	a	a	a	a
10	a	a	a	a
11	a	a	a	a
12	a	a	a	a
13	a	a	a	a
14	a	a	c	a
15	a	a	a	a
16	a	a	a	a
17	a	b	a	a
18	a	a	a	b
19	a	a	a	a
20	a	a	c	a
21	a	a	a	a
22	a	c	a	c
23	a	a	a	a
Sums	21a, 2c	19a, 3b, 1c	19a, 2b, 2c	18a, 2b, 3c

From this Table it is quite clearly revealed that there is practically no difference between quality of data at different concentrations of CO_2, and, that of the 92 plateaux, 83·7 p.c. are of "a" quality, 7·6 of "b" quality and 8·7 of "c" quality.

The means of the times in minutes taken to reach the plateaux, for the approximate 2, 4 and 6 p.c. classes, were respectively, 2·3 (ranging from 1·5 to 4), 2·4 (ranging from 1·5 to 4·5) and 1·9 (ranging from 1·0 to 2·5).

Statistical interpretation and evaluation.

Two curves were fitted to the data:

(1) $y = \text{antilog } (kx + 2) - 100.$

(2) $y = \text{antilog } (k_1x + k_2x^2 + 2) - 100.$

where y is the ventilatory increment expressed as a percentage, and x is the percentage of CO_2 in the inspired air.

The analysis of variance for the two curves (using the transformed values $z = \log (y + 100) - 2$) and the corresponding multiple correlation coefficients are given as below.

Analysis of variance (Curve 1).

Source	d.f.	S.S.	M.S.
Regression	1	9·1925	9·1925
Residual	68	·5186	·007626
Total	69	9·7741	

$R^2 = 9{\cdot}1925/9{\cdot}7741 = {\cdot}9405$

where *d.f.* = degrees of freedom.

S.S. = sums of squares.

M.S. = mean square.

R^2 = multiple correlation coefficient.

Analysis of variance (Curve 2).

Source	d.f.	S.S.	M.S.
Regression	2	9·6692	4·8346
Residual	67	·1049	·001566
Total	69	9·7741	

$$R^2 = 9{\cdot}6692/9{\cdot}7741 = {\cdot}9893.$$

The increase in multiple correlation coefficient for the second curve was tested in the following analysis.

Source	d.f.	S.S.	M.S.
Diff. in Regression	1	·4137	·4137
Error	67	·1049	·001566

$$F = 264{\cdot}17$$

From these analyses it can be seen that both curves fit the data reasonably well, and that the second curve gives relatively a much better fit than the first.

The second curve is shown in Fig. 3, and the effects of CO_2 concentrations, differing from each other by increments of 0·5 p.c. up to 7 p.c. derived from $y = \text{antilog } (k_1x + k_2x^2 + 2) - 100$, are given in Table 4.

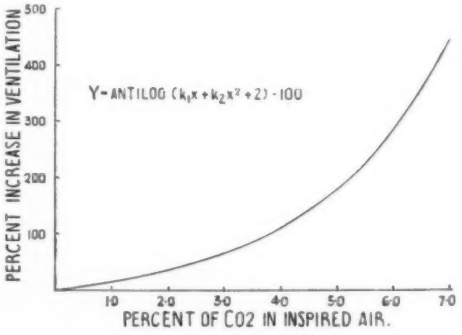

Fig. 3.

In order to show graphically the scatter of the response curves of individual subjects, the curve of each subject (fitted by eye) has been drawn, and all are incorporated in Fig. 4.

As an indication that for any individual subject the data represents a reliable picture of the response to CO_2 for that subject, five curves are shown in Fig. 5 (numbers 1, 5, 10, 15 and 20)[2]. The separate points from which each curve is drawn are shown in relation to that curve.

It is clear that the deviations of individual points from their respective curves are quite small.

DISCUSSION.

Literature on the subject of the quantitative response to CO_2 is scanty. While it has been known for a very long time that CO_2 in low concentrations has a stimulating effect upon ventilation, and in higher concentrations a toxic action is observed, yet, very little well-controlled experimental work upon the actual magnitude of the response to low concentrations has appeared.

[2] Since the curves were numbered in time sequence, the choosing of the numbers as multiples of five after the first gives a representative random sample.

All, or some, of three main criticisms may be levelled at most of the experimental work.

(1) Paucity of data due to too few subjects. (2) Use of a rebreathing method (i.e. closed circuit), in order continuously to raise the CO_2 percentage in the inspired air above that of the normal atmosphere. (3) No apparent assurance that the subject has in fact achieved a ventilatory plateau.

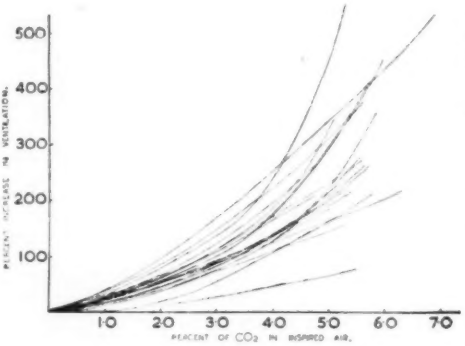

Fig. 4.

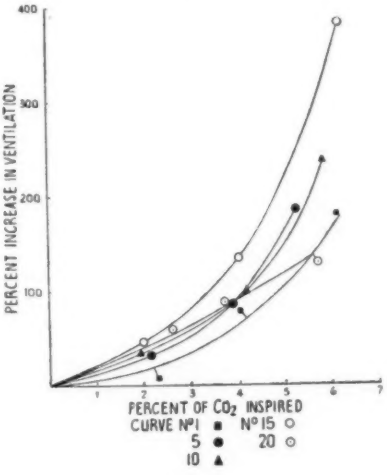

Fig. 5.

It is far better, of course, to use constant mixtures of CO_2 with air or oxygen, than to rely on rebreathing in a closed circuit. Investigators who use the latter method, collecting the air breathed for a known period and measuring its volume, and establishing its CO_2 concentrations at the beginning and end of the collection time, or a single value in the middle of the collection time, err, because they have failed to account for the shape of the CO_2 response curve, which is not a straight line slope. Also, in cases where the expired air is collected over a known time, and then measured, there is no way of knowing whether the ventilation rate has, indeed, been reasonably constant over this time, or whether it has risen or fallen significantly.

In Table 3 are set out the best of the quantitative investigations on CO_2 response the writer has been able to find. For convenience, it contains the percentage increases in ventilation rate, above the normal resting level, which different investigators have found for different concentrations of CO_2 in air or oxygen, together with the number of subjects employed in the investigation, and a remark on the general technique. The values have been obtained from Tables and, in some cases, from graphs.

TABLE 3.

Investigators	Results—Ventilatory increase p.c. above resting with various CO_2 percentages							No. of subjects	Remarks
	1 p.c.	2 p.c.	3 p.c.	4 p.c.	5 p.c.	6 p.c.	7 p.c.		
Shock and Soley	28·4 (2) 14·2 (1)	+ 52·8 (2) + 34·3 (1)		+ 128·5 (2) + 98·6 (1)					No. of subjects were: 1 p.c. class—17 subjects 2 p.c. class—12 subjects 4 p.c. class—15 subjects
Davies, Brow and Binger		+ 15	+ 45	+ 95	+ 190	+ 350		1	This study was done on only one subject and a closed circuit re-breathing method was used. Such a method is of doubtful reliability.
Heller, Killiches and Drinker					+ 168·6			22	In this apparently thorough study, a constant mixture of CO_2 in air was employed. The effects of a 7 p.c. mixture were also measured, but the results were not given.
Peabody	+ 7	+ 15	+ 40	+ 65	+ 110	+ 190	+ 520	9	Subjects of both sexes and various ages were used. Also, a re-breathing method was used. Values thus obtained are suspect, which Peabody himself realized.
Scott	+ 13	+ 38	+ 55	+ 85	+ 130	+ 200	+ 300	2	As only two normal subjects were used in this study it is merely of passing interest.

(1) and (2) refer to CO_2-air and CO_2-oxygen mixtures, respectively.

The two best studies appear to be those of Shock and Soley, and of Heller, Killiches and Drinker.

The former study has the fault that, although the subjects breathed mixtures of constant composition, no very exact method is mentioned for deter-

mining when a plateau has been reached. The authors merely state that "the (test) period was long enough for the respiratory volume to become stabilized". They do not state the time required for this.

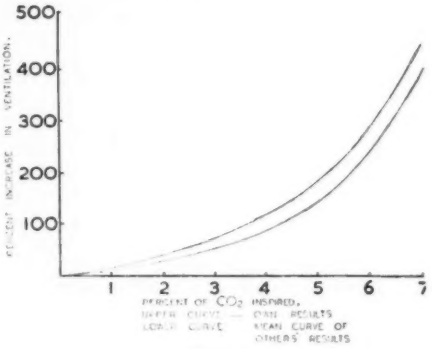

Fig. 6.

Heller, Killiches and Drinker, studying the effects of 5 and 7 p.c. CO_2, and using a more suitable system of measurement, were able to establish when plateaux had been reached, and they observe that the time required to reach a plateau varied from $2\frac{1}{2}$ to 10 minutes. They do not define a plateau.

The results obtained in this present investigation seem to agree fairly well with the values obtained by others and, because of the number of subjects (23) and the use of a "plateau" method of measurement, they are probably more reliable.

The results, which may be seen in Fig. 3 for the various CO_2 percentages, are brought together in Table 4.

In Fig. 6, the curve obtained in the present investigation is shown compared with that obtained by taking the means of the values published by others, as set out in Table 3.

TABLE 4.

CO_2 percentage in inspired air.	0·5	1	1·5	2·0	2·5	3·0	3·5	4·0	4·5	5·0	5·5	6·0	6·5	7·0
Percentage increment over resting ventilation rate.	6	14	23	34	48	65	85	110	140	178	224	282	355	446

More references could have been cited than have been, but it was felt that most of them would necessarily be of historical interest only, because of inaccurate and unsatisfactory measuring methods, or of the use of too few subjects.

SUMMARY.

The effects of low percentages of CO_2 in the inspired air on the ventilation of normal young men have been measured.

The methods of measurement and the appraisal of data have been explained, and the apparatus and experimental procedure have been described. The methods of measurement have been compared with those of other authors, and the advantages of the "plateau" method indicated.

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